REMARKS

Claims 17, 19-24, 28, 29, 31, 33, 38, 41, 43, 49 and 56-61 are pending. Claims 22, 57 and 58 are newly canceled without prejudice by Applicant. Claims 17, 19-21, 23-24, 28, 29, 31, 33, 34, 38, 41, 43, 49, 56 and 59-61 are emended to adjust claim dependencies. Claims 62-63 are newly added. No new matter has been entered.

35 U.S.C. 112, first paragraph, new matter

Claims 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 41, 42, 49, 56, 57, 58, 59, 60 and 61 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement.

The office action indicates that the recitation of the phrase "comprises leukocytes which have not been fractionated into cell types" in instant claims 57 and 58 in reference to the blood samples, is new matter. Applicant respectfully traverses for the reasons set forth in Applicants reply filed October, but solely in the interest of advancing prosecution, has cancelled claims 57 and 58, without prejudice. Applicant has also deleted the dependency of claims 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 41, 42, 49, 56, 59, 60 and 61 from newly cancelled claims 57 and/or 58, without prejudice.

In light of the above amendments to the instant claims, Applicant contends the rejection is moot.

35 U.S.C. 102

Claims 1, 19-21, 23-24, 28-29, 31, 33-34, 38, 41, 49, 56, 57, 58 and 61 are rejected under 102(a) and 102(b) as being anticipated by Ralph et al (WO 98/24935) and under 35 U.S.C. 102(e) as being anticipated by Ralph *et al.* (US6,190,857).

> Anticipation requires that the purported prior art reference disclose each and every limitation of the claim. *Atlas Powder Company et al. v. IRECO, Incorporated et al.*, 190 F.3d 1342, 1347 (Fed. Cir. 1999).

Applicant respectfully traverses the rejection of the claims on the grounds that Ralph *et al.* does not teach each and every limitation of the claims.

The Examiner asserts at page 5 of the office action that the limitation of the claims corresponding to detecting disease marker RNA in "*RNA of blood samples which have not been fractionated into cell types*" is anticipated by Ralph et al. on the grounds that Ralph et al. teaches detection of disease marker RNA in "*total RNA from peripheral blood*" at Col. 98, lines 5-6.

In particular, the Examiner contends that the recitation "*total RNA from peripheral blood*" signifies that Ralph et al. teaches the limitation of the claims corresponding to detecting disease marker RNA in "*RNA of blood samples which have not been fractionated into cell types*", in accordance with the Examiner's plain assertion that "*total RNA from peripheral blood*" **is** "*RNA of blood samples which have not been fractionated into cell types*" (Office Action, page 5). The Examiner further plainly asserts at page 15 of the Office Action that '*... applicant is arguing that Ralph et al. may have said "DNA-free total RNA from peripheral blood" but that they didn't mean as much*', clearly indicating that the Examiner considers the term "*total RNA from peripheral blood*" to have an unambiguous meaning so as to inherently correspond to "*RNA of blood samples which have not been fractionated into cell types*", in accordance with the Examiner's plain assertion at page 16 that the term "*total RNA from peripheral blood*" constitutes "*very clear language*".

Thus, the plain language of the basis for the rejections in the Office Action indicates that the Examiner considers that the phrase "*total RNA from peripheral blood*" must be interpreted as inherently corresponding to "*RNA of blood samples which **have not** been fractionated into cell types*", and cannot be interpreted as "*RNA of blood samples which **have** been fractionated into cell types*".

Applicant respectfully submits, however, that the Examiner's interpretation is incorrect, as evidenced by the literature of the art. Applicant wishes to direct the Examiner to the enclosed excerpts from the Clontech Blood Total RNA Catalog, which can be considered a particularly authoritative art reference by virtue of representing a major commercial supplier of "Human Blood Total RNA". The first page of the Clontech catalog excerpts clearly uses the term "*total RNA from blood*" (as well as the equivalent term "*blood Total RNA*") to refer to RNA isolated from different types of fractionated blood

cell types. As can be seen in the second and third pages of the catalog excerpts, such cell types particularly include "*peripheral blood mononuclear cells*". As can be seen in the third page of the Clontech catalog excerpts, the source of total RNA from fractionated mononuclear cells is explicitly referred to as "*Normal human blood; peripheral blood mononuclear cells...*", which is clearly equivalent to referring to RNA isolated from fractionated mononuclear cells as "*total RNA from peripheral blood*". Applicant wishes to further direct the Examiner to the enclosed abstract of the academic publication Yu *et al.* (page 3816, left column, last paragraph) which explicitly refers to total RNA of isolated peripheral blood mononuclear cells as "*Total RNA of peripheral blood*", which again is clearly equivalent to stating that RNA isolated from fractionated mononuclear cells corresponds to "*total RNA from peripheral blood*".

Applicant wishes to point out that the qualifier "*total*" in the phrase "*total RNA from peripheral blood*" is a universally employed term of art widely used to refer to undegraded RNA representative of all of the different types of RNA (e.g. polyA RNA or mRNA, tRNA, rRNA, small nuclear RNA, etc.) present in a given sample, often to distinguish all of the RNA which is present in a sample in an undegraded state, which is often difficult to achieve, from the polyA RNA (i.e. mRNA) component of the sample, which is often of particular interest. This is clearly taught in the enclosed excerpts of Molecular Cloning by Sambrook and Russell 3rd ed. (please refer, for example, to the underlined passages; as well as to the arrowed elements in Figure 7-1), which is a universally employed technical reference manual. Thus, the qualifier "*total*" is similarly not used by Ralph et al. to qualify the RNA as being isolated from a "total" sample, i.e. from "total blood" or "whole blood".

Thus, Applicant respectfully submits that the Examiner's pivotal contention that total RNA from peripheral blood is inherently RNA of blood samples which have not been fractionated into cell types, upon which the rejections are based, is plainly incorrect, and hence that, on this basis alone, the claims are not in fact anticipated by Ralph et al.

The Examiner further asserts at page 16 of the Office Action, in disputing Applicant's reasoning whereby the language "*total RNA from peripheral blood*" in Ralph et al. corresponds to RNA from fractionated mononuclear cells, that Applicant "*does not ever point to a direct*

statement in Ralph where they contradict themselves regarding their very clear language that "DNA-free total RNA from peripheral blood" was used in the confirmation RT-PCR examples where they clearly and directly state that DNA-free total RNA was used." The Examiner further asserts that "*No example that includes this language refers back to other examples where mononuclear cells were isolated, even though Ralph et al. is very careful in many instances throughout their disclosure to point to the mononuclear isolation methods when they were used*". The Examiner concludes that this weighs heavily on the side of reasoning that Ralph et al. would have referred back to a mononuclear cell RNA isolation method if they intended to point to such a method.

With regard to the Examiner's statement that Ralph et al. do not contradict themselves regarding their language that "*DNA-free total RNA from peripheral blood*" was used in the confirmation RT-PCR examples, as explained by Applicant above there is in fact no inherent contradiction in referring to RNA isolated from fractionated mononuclear cells as "*DNA-free total RNA from peripheral blood*". Secondly, Applicant respectfully disagrees with the Examiner's contention that Ralph et al. do not refer to the mononuclear cell isolation method that was used to obtain RNA in the confirmation RT-PCR examples. Namely, Ralph et al. explicitly and specifically teach that **an aliquot of RNA isolated from fractionated mononuclear cell (i.e. RNA obtained using vacutainer CPT tubes with Ficoll gradients) was set aside specifically for "*RT-PCR confirmation using the external standard method described below*"** (Column 62, section 4.9.1 as a whole and sentence starting at line 55 in particular). This "*RT-PCR confirmation using the external standard method*" performed using an aliquot of RNA from fractionated mononuclear cells which is explicitly set aside for this purpose is indeed described below section 4.9.1; namely at Column 64, section 4.9.3.3, as well as in section 4.11.3, as being RT-PCR confirmation performed by using beta-actin mRNA as the "external standard". It can be clearly shown as follows that the RT-PCR confirmation performed using "*DNA-free total RNA from peripheral blood*" (Col. 98, lines 5-6) in section 5.6.2 referred to by the Examiner in making the rejections was performed using RNA from fractionated mononuclear cells. Namely, section 5.6.2 states that RT-PCR confirmation was performed as described in section 4.11.3, and section 4.11.3 in turn states that RT-PCR confirmation was performed using beta-actin as an external control (Column 68, line 50), i.e. using "the external standard method" described in

section 4.9.3.3 for which an aliquot of RNA from fractionated mononuclear cells was set aside, as explicitly dictated at Column 62, sentence starting at line 55. It may be noted that section 4.11.3 clearly refers to the "*RT-PCR confirmation with an external standard*" described in section 4.9.3.3, however whereas section 4.9.3.3 provides a very short general introduction to this method, essentially briefly outlining the advantages and rationale of the method, section 4.11.3 clearly merely describes a specific implementation of the general method described in section 4.9.3.3. Namely, section 4.11.3 provides a specific "Experimental Results" type description of experiments reduced to practice according to the method described in section 4.9.3.3, i.e. by describing, for example, the specific experimental groups tested, the specific cDNA synthesis kit employed, the specific primers used, the specific thermal cycling parameters used, the specific agarose gel concentration used, and the specific target genes tested; none of these specific experimental parameters being addressed in general introductory section 4.9.3.3. Thus, section 4.11.3, corresponds to the "*RT-PCR confirmation using the external standard method*" performed using the aliquot of RNA isolated from fractionated mononuclear cells set aside for this purpose referred to in section 5.6.2.

Thus, it can be clearly shown that the RT-PCR confirmation performed using "*DNA-free total RNA from peripheral blood*" (Col. 98, lines 5-6) at Column 98, section 5.6.2 referred to by the Examiner in making the rejections was performed using an aliquot of RNA from fractionated mononuclear cells explicitly set aside for this purpose, as described at Column 62, sentence starting at line 55. Applicant submits that on this sole basis, as well as in combination with Applicant's demonstration above that RNA from isolated mononuclear cells can be generally referred to in the art as "*total RNA from peripheral blood*", the claims are not anticipated by Ralph et al.

The instant claims, recite methods of identifying markers in "RNA of blood samples which have not been fractionated into cell types....". However, the Office Action characterizes Ralph *et al.* as teaching:

> "that responses secondary to disease states may be reflected in changing patterns of ***leukocyte*** mRNA levels that correlate with the presence of the disease state", emphasis added, referring to Column 5, lines 27-33 of US6,190,857, (page 5 of office action).

Applicant notes that the method of the instant claims does not analyze changing patterns of ***leukocyte*** mRNA, as disclosed by Ralph *et al.* By reciting methods of identifying markers in "RNA of blood samples which have not been fractionated into cell types.." , the instant claims encompass RNA which is not limited to that obtained from leukocytes, and as such, necessarily contains additional cell types and material. Therefore, Ralph *et al.*'s teaching of leukocyte mRNA levels does not take into account the levels of marker RNA of blood samples which have not been fractionated into cell types, as required by the instant claims.

The Office Action also indicates that Ralph *et al.* teach:

> "the use of RT-PCR to ***identify*** two or more markers useful for diagnosing a disease, namely prostate or breast cancer, exemplifying this method for the detection of two transcripts referred to by Ralph et al as UC331 and UC332, these sequences are RNA encoded by each of two genes" emphasis added, referring to Example 5.6.2 and following, and Column 98 of US6,190,857, (page 5 of office action).

Applicant disagrees with the contention of the office action that Ralph *et al.* teaches "the use of RT-PCR to ***identify*** two or more markers useful for diagnosing a disease" in Example 5.6.2 and following. Applicant notes that Ralph *et al.* teach an independent ***confirmatory method*** in Example 5.6.2:

> "5.6.2 Relative Quantitative RT-PCR™
>
> Approximately 1.5-5.0 μg of DNA-free total RNA from the peripheral blood of healthy volunteers or patients with either metastatic prostate or breast cancer were analyzed by relative quantitative RT-PCR as described in section 4.11.3 above. Typically, the cDNA derived from the reverse transcription of 5.0 μg of RNA resulted in enough normalized cDNA to perform 50-200 RT-PCR™ reactions.
>
> The oligonucleotides used in the relative quantitative RT-PCR™ studies that ***independently confirmed the differential expression of UC331*** were designed from the sequence in the human UC331 virtual contig. These UC331 specific oligonucleotides had the sequences of 5' CTGGCCTIACGGAAGATACGACAC 3' (SEQ ID NO:25) and 5' ACAATCCGGAGGCATCAGAAACT 3' (SEQ ID NO:26). These oligonucleotides direct the amplification of a 277 nucleotide long PCR™ product that is specific for UC331.
>
> The oligonucleotides used in the relative quantitative RT-PCR™ studies that ***independently confirmed the differential expression of UC332*** were designed using the

> sequences of the cDNA with the GenBank accession number D87451. These UC332 specific oligonucleotides had the sequences 5' AGCCCCGGCCTCCTCGTCCTC 3' (SEQ ID NO:27) and 5' GGCGGCGGCAGCGGTTCTC 3' (SEQ ID NO:28). These oligonucleotides direct the amplification of a 140 nucleotide long PCR™ product that is specific for UC332", emphasis added, US6,190,857, column 98, lines 4-30,

Thus, Example 5.6.2 teaches a method of ***confirming*** a biomarker that has already been identified. In contrast, the instant claims are drawn to a method of ***identifying*** a biomarker, not confirming a biomarker that has already been identified.

Further, Applicant notes that the markers UC331 and UC332 taught by Ralph *et al.* were first ***identified*** as markers using a method which analyzed RNA from ***fractionated cells*** of peripheral blood:

> "5.6 Example 6
>
> Two mRNAs with Conserved Sequence Motifs are up Regulated in Nucleated Blood Cells of Patients with Metastatic Breast or Prostate Cancer
>
> ***RNA fingerprinting was performed as described in Section 4.12*** above. Two additional differentially appearing PCR™ amplified cDNA fragments ***were identified*** in these studies, labeled as UC331 and UC332", emphasis added, US6,190,857, Column 95, lines 32-39" emphasis added, US6,190,857,

and

> "**4.12** ***Identification*** of UC 331 and UC 332 "
>
> "4.12.2 RNA Fingerprinting
>
> ***RNA was prepared as described in section 4.11.1 above***. RNA fingerprinting with arbitrarily chosen oligonucleotide primers (Welsh *et al.*, 1992) was performed as described in section 4.11.2, with certain modifications", emphasis added, US6,190,857, Column 69, line 64, and column 70, lines 8-20.

and

> "**4.11** Detection and Diagnosis of Metastatic Breast and Prostate Cancer
>
> 4.11.1 Preparation of RNA
>
> ..... ***RNA was prepared*** from nucleated circulating peripheral blood cells as ***described in Section 4.9.1*** above...", emphasis added, US6,190,857, column 67, lines 1-3, and lines 12-13.

and

"4.9 Materials and Methods

4.9.1 Application of RNA Fingerprinting to Discover Biomarkers for Disease States

RNA fingerprinting (according to Liang and Pardee, 1992; Welsh *et al.*, 1992; Liang and Pardee, 1993) was applied to nucleic acids isolated from the peripheral blood of individuals with metastatic prostate cancer, compared with normal individuals.

Blood was drawn from cancer patients and normal individuals into Vacutainer CPT tubes ***with ficoll gradients*** (Becton Dickinson and Company, Frankin Lanes, N.J.). The tubes were centrifuged to separate the red blood cells from various types of nucleated cells, collectively referred to as the buffy coat, and from blood plasma. Total cell RNA was isolated from the buffy coats by the RNA STAT-60 method ....."

The above excerpts from Ralph *et al.* make it clear that their biomarkers UC331 and UC332 were first ***identified*** using a method which analyzed RNA from ***fractionated cells*** of peripheral blood, which is in contrast to RNA from blood samples which have not been fractionated into cell types, as required by the instantly claimed method. Because Ralph *et al.* does not disclose each and every limitation of these claims, it does not qualify as an anticipatory reference under 35 U.S.C. 102 (a) (b) or (e).

The Office Action further indicates that Ralph *et al.* teach :

"using an oligonucleotide of predetermined sequence which are primers specific to the particular transcripts to detect a presence of the RNA molecules", referring to column 98, lines 17-19 and 26-27 of US6,190,857, (page 5 of office action).

The referenced primers of predetermined sequence were described and used in Example 5.6.2 printed on Column 98. As discussed above, Applicant notes that Example 5.6.2 of Ralph *et al.* teaches that these sequence specific primers were used in a method ***to confirm the identity*** of the biomarkers UC331 and UC332, not in a method to ***identify*** the UC331 and UC332 biomarkers, as required by the instant claims. Further, Applicant notes that the markers UC331 and UC332 were ***identified*** using primers which were not specific to UC331 and UC332 transcripts:

"5.6 Example 6

Two mRNAs with Conserved Sequence Motifs are up Regulated in Nucleated Blood Cells of Patients with Metastatic Breast or Prostate Cancer

RNA fingerprinting was performed as described in Section 4.12 above. Two additional differentially appearing PCR™ amplified cDNA fragments were identified in these studies, labeled as UC331 and UC332. ***UC331 was identified using a first promiscuous primer*** with the sequence 5'-ACGACTCACTATAAGCAGGA-3' (SEQ ID NO:13). The second promiscuous primer used was 5'-AACAGCTATGACCATCGTGG-3' (SEQ ID NO:23). ***UC332 was identified using a first promiscuous primer*** with the sequence 5'-ACGACTCACTATGTGGAGAA-3' (SEQ ID NO:24). The second promiscuous primer used was 5'-AACAGCTATGACCCTGAGGA-3' (SEQ ID NO:52).

and

"4.12 Identification of UC 331 and UC 332

4.12.2 RNA Fingerprinting

RNA was prepared as described in section 4.11.1 above. RNA fingerprinting with arbitrarily chosen oligonucleotide primers (Welsh *et al.*, 1992) was performed as described in section 4.11.2, with certain modifications. In Example 7, the strategy of RNA fingerprinting used was similar to that described in Ralph *et al.* (1993) except that oligonucleotide primers used were composed of two discrete domains. ***The 5' domain of these oligonucleotides consisted of ten nucleotides that complemented sequences from either the T7 promoter or the M13 reverse sequencing primer. The 3' domains of these oligonucleotides were 8-mer sequences predicted to anneal frequently to the protein-coding regions of mRNAs in a promiscuous fashion*** (Lopez-Nieto and Nigam, 1996). These oligonucleotides were then used in a sequential pairwise strategy that optimizes the amount of mRNA complexity that can be surveyed with limited numbers of primers and starting RNA", emphasis added,

Thus, Ralph *et al.* teaches a method of ***identifying*** UC331 and UC332 markers using ***promiscuous probes***, which is in direct contrast to the limitation of the instant claims which require the use of a probe which is specific for the biomarker RNA or cDNA thereof. Further, Ralph *et al.* teaches a method of ***identifying*** UC331 and UC332 markers using RNA which had has been isolated from blood cells fractionated by a Ficoll gradient, which is in contrast to the limitation of the instant claims which require the use RNA of blood samples which have not been fractionated into cell types. Because Ralph *et al.* does not disclose each and every

limitation of these claims, it does not qualify as an anticipatory reference under 35 U.S.C. 102 (a) (b) or (e).

Claims 57 and 58, and their claim dependencies have been cancelled without prejudice, rendering their rejection moot.

In light of these remarks and claim amendments, Applicant respectfully requests reconsideration and withdrawal of the rejection of the instant claims.

Claim Rejections – 35 USC § 103

Claim 43 is rejected under 35 U.S.C. 103(a) as being unpatentable over Ralph *et al.* (US 6109857 and WO 98/24953) in view of Sharma *et al.* (WO 98/49342).

> To establish prima facie obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974).

Applicant respectfully traverses that the instant claims are rendered obvious by Ralph *et al.* in view of Sharma *et al.* on the grounds that Ralph *et al.* does not teach all the limitations required by the instant claims and the teachings of Sharma *et al.* do not make up the difference.

As discussed above, Ralph *et al.* teaches a method of identifying UC331 and UC332 markers using ***promiscuous probes***, which is in direct contrast to the limitation of the instant claims which require the use of a probe which is specific for the biomarker RNA or cDNA thereof. Further, Ralph *et al.* teaches a method of identifying UC331 and UC332 markers using ***RNA from cells which has been fractionated by a Ficoll gradient***, which is in contrast to the limitation of the instant claims which require the use RNA of blood samples which have not been fractionated into cell types. As acknowledged in the office action, Ralph *et al.* does not teach applying their disclosed methods to identify markers for colorectal cancer.

The Office Action contends that Sharma *et al.*'s teaching of a method useful for diagnosing a disease by looking for differentially expressed genes in total RNA isolated from whole blood samples, and their proposal that their method would be useful in the disease of

cancer of the bowel, provide motivation to one of skill to have modified the methods taught by Ralph *et al.* so as to have screened for markers for colorectal cancer as taught by Sharma *et al.*

However, the motivation provided by Sharma to apply the method of identifying markers for disease taught by Ralph *et al.*, to the disease of colorectal cancer, is not sufficient to remedy the failure of Ralph *et al.* to teach all of the limitations of the instantly claimed methods, specifically the failure to teach the limitation of identifying markers using an oligonucleotide which is specific for the marker RNA or cDNA thereof, and also the limitation that requires the use of RNA of blood samples which have not been fractionated into cell types.
In light of the claim amendments and remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejection.

Claims 17, 19, 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 41, 43, 56, 57, 58 and 61 are rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* (WO 98/49342) in view of Ralph *et al.* (US 6109857 and WO 98/24953).

Applicant respectfully traverses that the instant claims are rendered obvious by Sharma *et al.* in view of Ralph *et al.* on the grounds that Sharma *et al.* does not teach all the limitations required by the instant claims and the teachings of Ralph *et al.* do not make up the difference.

As acknowledged in the office action, and as discussed above, Sharma *et al.* does not teach a method of identifying two or more biomarkers comprising use of an oligonucleotide of predetermined sequence specific for the biomarker as required by the instant claims. The Office Action indicates that Ralph *et al.* teach:

> " a method of ***identifying*** differentially expressed markers using RNA fingerprinting.....using random primers and ***identifying*** differentially expressed molecules using gel electrophoresis", emphasis added, page 10 of office action.

Applicant notes that the instant claims recite an entirely different set of method steps for ***identifying*** a marker from that taught by either Ralph *et al.* or Sharma *et al.*, with a pointed difference being that the instant claims require the use of an oligonucleotide probe that is specific

for a biomarker ***in the first step*** of the method.

The office action further indicates that Ralph *et al.* explicitly teach:

> "that frequently mRNAs identified by RNA fingerprinting or differential display as being differentially regulated turn out not to be so when examined by independent means. It is critical that the differential expression of all mRNAs identified by RNA fingerprinting ***be confirmed*** as such by an independent methodology", emphasis added, page 10 of office action.

and contends that:

> ".... it would have been obvious to one of skill in the art at the time the invention was made to have modified the methods taught by Sharma *et al.* so as to have included the RT-PCR step using oligonucleotides of predetermined sequence as taught by Ralph *et al.* so as to have provided a means to ***confirm the differential expression of the identified markers***", page 11 of office action.

The office action indicates that Ralph *et al.* exemplify this confirmation method in Example 5.6.2 using RT-PCR.

As discussed above, Applicant notes that unlike the method taught by Ralph *et al.* in identifying UC331 and UC332, the instantly claimed methods of identifying markers do not recite the use of RNA fingerprinting using non specific probes. In contrast, step (a) of the instantly claimed methods of ***identifying markers*** explicitly recites the use of an oligonucleotide which is specific only for the marker RNA or cDNA thereof. Therefore, Ralph *et al.*'s teaching of an independent, ***confirmatory*** RT-PCR method to confirm a method that ***identifies markers UC331 and UC332 using promiscuous probes***, is not applicable to the instantly claimed, sequence-specific ***method which specifically recites a method of identifying markers.***

Further, Ralph *et al.*'s teaching of a secondary method to confirm markers that have already been identified, which may optionally encompass RT-PCR with sequence specific probes, is an independent method that is ***supplemental to*** and distinct from methods which identify markers. Though these independent confirmatory methods may be useful, especially to supplement methods of identifying markers based on promiscuous probes, they are not required by the instantly recited claims, nor can non-recited limitations be incorporated into the instant claims.

Because neither the Sharma *et al.* nor Ralph *et al.*, when each is considered either alone or in combination with each other, teach all the limitations of the instant claims, specifically the limitation that a sequence specific probe be used in a method of identification of a marker, the claims are not rendered obvious by the teachings of these cited references.

Applicant's cancellation, without prejudice, of claims 57 and 58, renders the rejection of newly canceled claims 57 and 58 moot.

In light of the claim amendments and remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejection.

Claim 59 is rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* (WO 98/49342) in view of either Ralph *et al.* (US 6109857 and WO 98/24953) as applied to claims 17, 19, 57 and 58, and further in view of Wei *et al.*

Applicant has newly amended claim 59 to remove its dependency from newly cancelled claims 57 and 58. The traversal is therefore based on the application of the above cited references to claims 17 and 19.

The office action contends:

> " that it would have been prima facie obvious to one of ordinary skill in the art to have applied the methods taught by Sharma *et al.* in view of Ralph *et al.* to the disease diabetes in order to identify additional markers in blood that would be useful for detecting and understanding this disease.

Applicant respectfully traverses on the grounds discussed above with respect to independent claims 17 and 19, specifically that neither the Sharma *et al.* nor Ralph *et al.*, when each is considered either alone or in combination with each other, teach all the limitations of the instant claims, particularly a method of identifying two or more markers for diabetes comprising the limitations that a ***sequence specific probe*** be used in a method of ***identification*** of a marker in analyzing blood samples ***which have not been fractionated into cell types***.

The office action acknowledges that neither Sharma *et al.* nor Ralph *et al.* teach using their methods for the detection of diabetes, in particular.

Wei *et al.* teach that IL-6 mRNA is expressed in higher amounts in peripheral blood

mononuclear cells from patients with Insulin Dependent Diabetes Mellitus (IDDM) relative to blood of normal individuals, but is silent with respect to the differential expression, if any, in RNA from blood samples which have not been fractionated into cell types. Accordingly, Applicant respectfully contends that one of skill at the time the invention was made would not have a reasonable expectation of success in identifying IL-6 as a marker for diabetes in blood samples which have not been fractionated into cell types, as required by the instant claims.

Further, Wei *et al.* is silent with respect to the differential expression of a second RNA molecule in peripheral blood mononuclear cells of patients with IDDM relative to normal individuals. Accordingly, Applicant respectfully contends that one of skill at the time the invention was made would not have a reasonable expectation of success in identifying a second RNA as a marker for diabetes in blood samples which have not been fractionated into cell types, as required by the instant claims. Neither Ralph *et al.*, nor Sharma *et al.*, nor Wei *et al.* provide one of skill at the time of the invention a scientific basis for a reasonable expectation of success in arriving at the claimed invention of identifying two or more markers for diabetes in whole blood using the claimed methods.

Thus Applicant disagrees with the contention in the Office Action that given the teachings of Sharma *et al.* and Ralph *et al.* one of skill would have reasonably expected to identify numerous additional markers in RNA extracted from whole blood using differential display methods, and then to have confirmed those markers using RT-PCR.

Further, Applicant notes that the instantly claimed methods are directed to a **method of identifying markers for identifying two or more diabetes markers,** and not to an independent method of confirming a marker already identified as a marker for diabetes. As discussed above, Ralph *et al.*'s teaching of the use of RT-PCR as an independent confirmatory method to confirm a method that identified markers UC331 and UC332 with promiscuous probes in Example 5.6.2, as cited in the office action, is not applicable to the instantly claimed, sequence-specific method of identifying markers. Applicant further notes this inapplicability is especially inconsistent with the RT-PCR step (a) of instant independent claim 19 from which the instant claim depends.

Therefore, Applicant respectfully contends that neither the Sharma *et al.* nor Ralph *et al.*, nor Wei *et al.*, when each is considered either alone or in combination with each other, teach all the limitations of the instant claims, particularly a method of identifying two or more markers for

diabetes comprising the limitations that a ***sequence specific probe*** be used in a method of ***identification*** of a marker for diabetes in analyzing blood samples ***which have not been fractionated into cell types***.

In light of the claim amendments and remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejection.

Claim 60 is rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* (WO 98/49342) in view of either Ralph *et al.* (US 6109857 and WO 98/24953) as applied to claims 17, 19, 57 and 58, and further in view of Kasuga *et al.*

Applicant has newly amended claim 59 to remove its dependency from newly cancelled claims 57 and 58. The traversal is therefore based on the application of the above cited references to claims 17 and 19.

The office action contends:

> " that it would have been prima facie obvious to one of ordinary skill in the art to have applied the methods taught by Sharma *et al.* in view of Ralph *et al.* to the disease of heart failure in order to identify additional markers in blood that would be useful for detecting and understanding this disease", office action page 13.

Applicant respectfully traverses on the grounds discussed above with respect to independent claims 17 and 19, specifically that neither the Sharma *et al.* nor Ralph *et al.*, when each is considered either alone or in combination with each other, teach all the limitations of the instant claims, particularly a method of identifying two or more markers for heart failure comprising the limitations that a ***sequence specific probe*** be used in a method of ***identification*** of a marker in analyzing blood samples ***which have not been fractionated into cell types***.

The office action acknowledges that neither Sharma nor Ralph teach using their methods for the detection of heart failure, in particular.

Kasuga et al teaches that the expression of monocyte chemotactic and activating factor mRNA is known to increase in the blood of acute heart failure patients.

Applicant disagrees with the contention in the Office Action that given the teachings of Sharma *et al.* and Ralph *et al.* one of skill would have reasonably expected to identify numerous

additional markers in RNA extracted from whole blood using differential display methods, and then to have confirmed those markers using RT-PCR.

Further, Applicant notes that the instantly claimed methods are directed to a **method of identifying markers for identifying two or more heart failure markers,** and not to an independent method of confirming a marker already identified as a marker for heart failure. As discussed above, Ralph *et al.*'s teaching of the use of RT-PCR as an independent confirmatory method to confirm a method that identified markers UC331 and UC332 with promiscuous probes in Example 5.6.2, as cited in the office action, is not applicable to the instantly claimed, sequence-specific method of identifying heart failure markers. Applicant further notes this inapplicability is especially inconsistent with the RT-PCR step (a) of instant independent claim 19 from which the instant claim depends.

Therefore, Applicant respectfully contends that neither the Sharma *et al.* nor Ralph *et al.*, nor Kasuga et al., when each is considered either alone or in combination with each other, teach all the limitations of the instant claims, particularly a method of identifying two or more markers for heart failure comprising the limitations that a ***sequence specific probe*** be used in a method of ***identification*** of a marker for heart failure in analyzing blood samples ***which have not been fractionated into cell types***.

In light of the claim amendments and remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejection.

Double Patenting

The office action indicates that the previously set forth rejections of obviousness type double patenting are maintained and applied to claims 57-61. Applicant respectfully traverses the rejection, but will consider filing a terminal disclaimer upon allowance.

Conclusion

Applicant submits that all claims are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with

Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Date: June 20, 2008

Respectfully submitted,

Amy DeClemp 54849 for

Name: Kathleen Williams
Registration No.: 34,380
Customer No.: 21874
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P.O. Box 55874
Boston, MA 02205
Tel: 617-239-0100

Encl:

Clontech Catalog – Human Blood Total RNA

Yu *et al.*, 2007.

Sambrook et al.

Clontech

Human Blood Total RNA, Assorted Cell Types

- *The highest quality Total RNA from blood.*
- *Choose RNA from a list of various blood cell types.*
- *Inquire to confirm availability.*

more

You may also be interested in these Clontech products:

AdvantageS HD DNA Polymerase Mix
Poly A+ RNA, Miscellaneous
Poly A+ RNA, Rat
Poly A+ RNA, Mouse
Total RNA, Miscellaneous
Total RNA, Rat
Total RNA, Mouse
Total RNA, Human
Poly A+ RNA, Human
Universal Reference Total RNA
SMART™ PCR cDNA Synthesis Kit

Product Name	Size	Catalog Number	Price	Add to Cart
Human Blood Total RNA, Assorted Cell Types	25 µg	636589	$263.00 USD	

Clontech

Human Blood Total RNA, Assorted Cell Types

Choose one item from the following list of blood Total RNA products, call first to confirm availability:

CD4+ activated (Lot Number 6090296)
CD4+ resting (Lot Number 6090289)
CD8+ activated (Lot Number 6090294)
CD14+ resting (Lot Number 6090290)
CD19+ activated (Lot Number 6090295)
CD19+ resting (Lot Number 6090293)
Peripheral blood mononuclear cells, activated (Lot Number 6090291)

Total RNA from individual blood cell types are sold separately, call to confirm availability of RNA from your blood cell type of choice.

You may also be interested in these Clontech products:

Advantage® HD DNA Polymerase Mix
Poly A+ RNA, Miscellaneous
Poly A+ RNA, Rat
Poly A+ RNA, Mouse
Total RNA, Miscellaneous
Total RNA, Rat
Total RNA, Mouse
Total RNA, Human
Poly A+ RNA, Human
Universal Reference Total RNA
SMART™ PCR cDNA Synthesis Kit

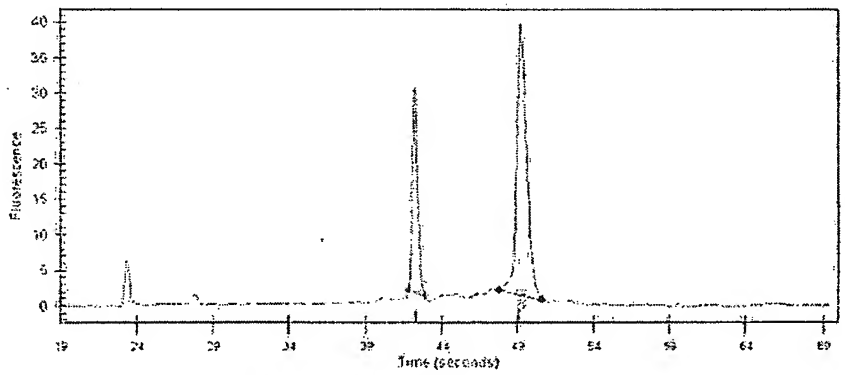


Figure 1. To confirm highest quality, all lots of Human Blood Total RNA are analyzed by capillary electrophoresis using an Agilent 2100 Bioanalyzer. Above is a trace of Total RNA from CD19+ activated normal human blood cells. For data from individual lot numbers refer to the respective certificates of analysis (CofA).

Product Name	Size	Catalog Number	Price	Add to Cart
Human Blood Total RNA, Assorted Cell Types	25 µg	636589	$283.00 USD	

PRODUCT: Human Blood Total RNA, Assorted Cell Types

CATALOG No.
636589

CONCENTRATION
1 µg/µl

LOT NUMBER 6090291

FORM
Suspension of total RNA in DEPC-treated water.

STORAGE CONDITIONS
Store at –70°C

SHELF LIFE
1 year from date of receipt under proper storage conditions.

SHIPPING CONDITIONS
Dry ice (–70°C)

FOR RESEARCH USE ONLY

DESCRIPTION
Total RNA isolated by a modified guanidinium thiocyanate method (1).

PACKAGE CONTENTS
25 µg Total RNA from the blood cells specified below

TOTAL RNA SOURCE
Normal human blood; peripheral blood mononuclear cells (activated), pooled from 4 male/female Caucasians, ages: 38-54

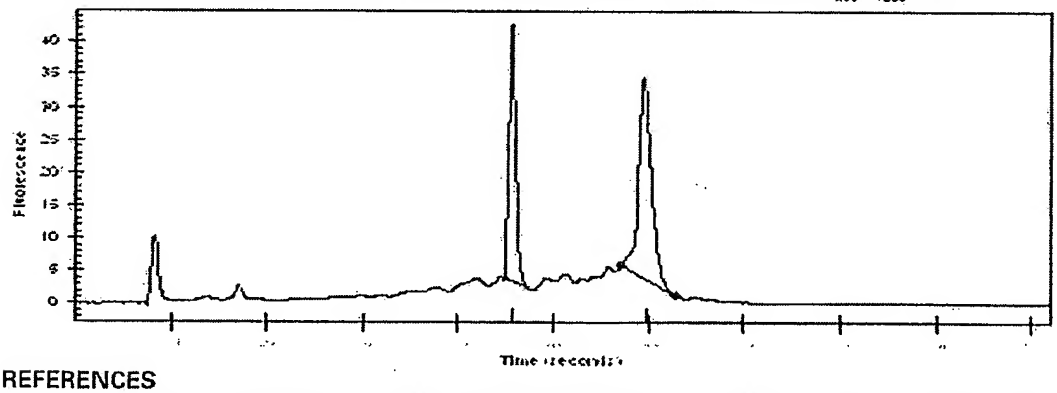
No further RNA source information is available.

IMPORTANT NOTE
To prevent contamination by RNases, always wear gloves when handling RNA. Avoid multiple freeze/thaw cycles.

QUALITY CONTROL DATA

This lot of total RNA was analyzed by capillary electrophoresis (CE) using an Agilent 2100 Bioanalyzer. The actual electropherogram trace for this RNA is provided below. RNA concentration and purity were evaluated by UV spectrophotometry.
Both the area ratio of the 28S/18S rRNA peaks, and the proportion (relative percentage) of these two peak areas to the total area under the electropherogram provide reliable quantitative estimates of RNA integrity. For both of these criteria, this sample meets or exceeds Clontech standards for high quality total RNA.

Peak Areas: 28S: 20.9 % 18S: 16.4 % Ratio 28S/18S: 1.3 Ratio A_{260}/A_{280}: 2.0



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1. Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

APPROVED BY:

(PA6X2114)

Identification and Clinical Significance of Mobilized Endothelial Progenitor Cells in Tumor Vasculogenesis of Hepatocellular Carcinoma

Decai Yu,[1,2] Xitai Sun,[1,2] Yudong Qiu,[1,2] Jianxing Zhou,[2] Yafu Wu,[2] Lingyuan Zhuang,[1] Jun Chen,[1] and Yitao Ding[1,2]

Abstract

Purpose: To investigate the distribution, frequency, and clinical significance of mobilized endothelial progenitor cells (EPC) in hepatocellular carcinoma (HCC).

Experimental Design: In healthy controls and patients with HCC, the frequency of circulating EPCs was determined by colony-forming assays, fluorescence-activated cell sorting, and real-time PCR. One hundred sixty-five–amino acid form of vascular endothelial growth factor and platelet-derived growth factor-BB in plasma and tissue were quantified by ELISA. The distribution and frequency of EPCs were evaluated by immunofluorescence, immunohistochemistry, and real-time PCR in normal liver ($n = 8$), and tumor tissue (TT), adjacent nonmalignant liver tissue (AT), and tumor-free tissue 5 cm from the tumor edge (TF), from 64 patients with HCC. Clinicopathologic data for these patients were evaluated.

Results: Compared with values for healthy controls, colony-forming unit scores were higher in the peripheral blood of patients with HCC. Plasma 165-amino acid form of vascular endothelial growth factor and platelet-derived growth factor-BB correlated with the expression level of the *AC133* gene, which was also higher in the peripheral blood of patients with HCC. Immunohistochemical analysis showed that EPCs were incorporated into the microvessels in cirrhotic and tumor tissue. Compared with normal liver (9.00), increased $AC133^+$ microvessel density (microvessels/0.74 mm^2) was found in TT (53.56), AT (84.76), and TF (48.33). The levels of *AC133* gene expression and AC133-microvessel density in AT, which were the highest among four groups, correlated with clinicopathologic variables (the absence of tumor capsule, venous invasion, proliferating cell nuclear antigen intensity, and early recurrence).

Conclusions: Mobilized EPCs participate in tumor vasculogenesis of HCC. *AC133* gene or antigen in peripheral blood and liver tissue could be used as a biomarker for predicting the progression of HCC.

Angiogenesis, the formation of new capillaries from preexisting vasculature, is essential for tumor growth and metastasis and represents an important prognostic indicator in hepatocellular carcinoma (HCC; ref. 1). Recent evidence suggests that endothelial cells from neighboring preexisting capillaries are not the only source of increased tumor vascularization. Bone marrow–derived endothelial progenitor cells (EPC) are also thought to contribute to the formation of new vessels in tumors, a process known as vasculogenesis (2). EPCs resemble embryonic angioblasts, which characteristically migrate, proliferate, and differentiate into mature endothelial cells (3). In general, EPCs can be identified as cells that simultaneously express the cell surface markers CD34, AC133/CD133, and kinase insert domain-containing receptor (4, 5).

Arbab et al. (6) and Shirakawa et al. (7) used mouse tumor models to show that bone marrow–derived EPCs are involved in tumor vasculogenesis and tumor growth, especially in early phases. In clinical investigations, two studies have reported that EPCs are recruited and homed with high specificity to solid tumors (8, 9). Reports on the numerical contribution of EPCs to vessel growth are variable, ranging from low (<0.1%) to high (up to 50%), likely dependent on the type of angiogenesis model used (10, 11). Moreover, unselected bone marrow cells (12) and endothelial progenitor-like cells (13) were engineered as vectors to hinder tumor angiogenesis and slow the growth of tumors. Because these conclusions support the hypothesis that EPCs play a functional role in vasculogenesis and growth of human solid tumors, there are possibilities that EPCs can be used as diagnostic or prognostic markers and as vectors for targeting cancers (11, 14).

HCC is a highly vascularized tumor. The majority of HCC tissue samples exhibit strong expression of proangiogenic

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factors, such as the 165-amino acid form of vascular endothelial growth factor ($VEGF_{165}$), platelet-derived growth factor-BB (PDGF-BB), insulin-like growth factor II, and basic fibroblast growth factor (15–18). These proangiogenic factors are important in the neovascularization, growth, and development of human HCC. These factors are also involved in activating, mobilizing, and recruiting EPCs from the bone marrow (19) and in promoting differentiation of EPCs into endothelial cells in some ischemic and tumor diseases (20, 21).

Recently, Poon et al. (22) reported that the level of circulating EPCs was elevated in patients with HCC and might correlate with the aggressiveness of the tumor. This was shown by a short-term culture assay involving scoring of colony-forming units (CFUs) of EPCs in the peripheral blood. Furthermore, there were positive correlations between the number of circulating EPCs and serum α-fetoprotein, VEGF, and interleukin-8 levels. However, there is no report providing evidence that EPCs participate in the neovascularization of HCC. Of note, animal models and clinical investigations have revealed that bone marrow–derived cells participate in neovascularization by committing to sinus endothelial cells in liver regeneration resulting from exposure to CCl_4 or from partial hepatectomy (23, 24).

Therefore, we hypothesized that EPCs were mobilized from the bone marrow of HCC patients by proangiogenic factors ($VEGF_{165}$ and PDGF-BB) and sustained the increase of vasculogenesis in HCC. We examined the number of EPCs in the peripheral blood and the distribution of EPCs in the liver of patients with HCC. We also analyzed the relationship between the level of *AC133* gene expression in liver and various clinicopathologic variables.

Materials and Methods

Patients and samples

Between January 2004 and August 2006, 64 patients were enrolled in the study in the Department of Hepatobiliary Surgery of Drum Tower Hospital. None of the patients had received preoperative treatment. Preoperative clinical and laboratory data, including routine liver biochemistry, a complete blood count, hepatitis B virus infection, and serum α-fetoprotein level, were prospectively assembled for each patient in a computerized database. Before surgery, peripheral blood was collected in EDTA-containing tubes through 21-gauge needles for colony-forming assays, fluorescence-activated cell sorting, plasma preparation, and RNA isolation. The peripheral blood from 20 healthy male volunteers (median age, 45 years; range, 38-59 years) and normal liver tissues from 4 liver transplant donors and 4 patients without liver disease served as healthy controls (HC). The research ethics committee of our hospital approved the protocol and informed consent was obtained from all participants.

Proangiogenic factors, such as VEGF (25) and hepatic growth factor (26), have higher expression in the surrounding liver than in the tumor itself, which are involved in mobilizing and recruiting EPCs (20, 21). Therefore, three different types of tissues from each HCC patient were assembled immediately after surgical resection: tumor-free tissue >5 cm far from the tumor edge (TF), adjacent nonmalignant tissue within 2 cm (AT), and tissue from the tumor (TT). Areas of tissue necrosis and hemorrhage were excluded. All of the tissue samples were snap frozen immediately after resection and kept in liquid nitrogen until they were used for experiments. Histopathologic examination of all specimens was done by a senior pathologist with experience in HCC pathology (Prof. L.H. Zhang), who was unaware of the preoperative clinical data and immunostaining results. Tumors were graded according to the criteria described by Edmonson and Steiner (27). Serial sections of the tumors and surrounding liver were examined to identify any tumor encapsulation, microscopic venous invasion, and microsatellite lesions. The degree of HCC invasiveness was verified according to the invasiveness scoring system for HCC (28).

EPC colony-forming assay

Peripheral blood mononuclear cells from HC and patients with HCC were suspended in EGM-2 MV BulletKit (CC-3202, Clonetics/BioWhittaker). After 48 h, nonadherent cells were collected and plated onto six-well plates coated with fibronectin (Sigma). The culture medium was changed every 3 days. On the 7th day, EPCs were identified with an inverted fluorescent microscope (Carl Zeiss, Inc.) by the uptake of 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Molecular Probes) and the binding of FITC-labeled Ulex europaeus agglutinin I (Sigma; ref. 29). CFUs, defined in the manner of Hill et al. (30) as consisting of multiple thin, flat cells emerging from a cluster of round cells, were quantified on the 7th day in a minimum of six fields (original magnification, ×40) per well by two observers without knowledge of the subjects' clinical profiles.

Fluorescence-activated cell sorting analysis

Circulating EPCs were measured by fluorescence-activated cell sorting analysis in unselected peripheral blood cells from HC and patients with HCC. EDTA-anticoagulated blood (100 μL) was incubated for 30 min at 4°C with 5 μL phycoerythrin-conjugated anti-AC133 (Miltenyi Biotec), PerCP-conjugated anti-CD45, and FITC-conjugated anti-CD34 (B&D). IgG1-FITC and IgG2α-phycoerythrin antibodies (B&D) served as isotype controls for each procedure. After incubation, cells were lysed and washed with PBS before analysis. For each sample, a minimum of 50,000 events was acquired. Circulating $CD34^+$ and $CD133^+$ mononuclear cells were tentatively classified as EPCs. Quantitative fluorescence analysis was done using FACSCalibur and WinMDI software (B&D).

Immunofluorescent staining of tissue sections

Frozen liver tissue sections were cut to a thickness of 4 or 20 μm. Sections were fixed in cold acetone for 5 min, air dried, and immersed in PBS. Sections were incubated with phycoerythrin-conjugated anti-AC133 (1:100) and FITC-conjugated anti-CD34 (1:100) at 37°C for 2 h. PBS was substituted for antibodies as a negative control. Fluorescently labeled cells were detected in the vessel wall via a fluorescence microscope using a Zeiss Axiovert scope (Carl Zeiss) and confocal laser scanning microscopy (MRC 1024, Bio-Rad, Inc.). A series of 1-μm optical sections through the entire thickness of the tissue were obtained using a 40× objective, and Z series were constructed from these sections.

Immunohistochemistry of frozen sections. Consecutive frozen liver tissue sections of 4-μm thickness were cut in a cryostat, fixed, and incubated with AC133 (1:100; Miltenyi Biotec) or CD34 (1:300; Santa Cruz Biotechnology) monoclonal mouse anti-human antibody monoclonal antibodies at 4°C overnight. A subsequent reaction was done with biotin-free horseradish peroxidase enzyme–labeled polymer from an EnVision plus detection system (DAKO). Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Negative controls were obtained by substituting the primary antibodies with PBS.

Immunohistochemistry of paraffin-embedded sections. Conventionally processed and embedded sections cut at a thickness of 4 μm were deparaffinized, blocked, and incubated at 4°C overnight with anti–proliferating cell nuclear antigen (PCNA) antibody (DAKO). A subsequent reaction was done as described above.

Determination of $CD34^+$ and $AC133^+$ microvessel density. Microvessel density (MVD) was evaluated according to Gasparini's criteria (31) on an Olympus microscope (C-X31) with an Olympus camera (C-5050Z) by two independent observers who were blinded to the patients' clinicopathologic data. Any brown-stained $CD34^+$ cells or

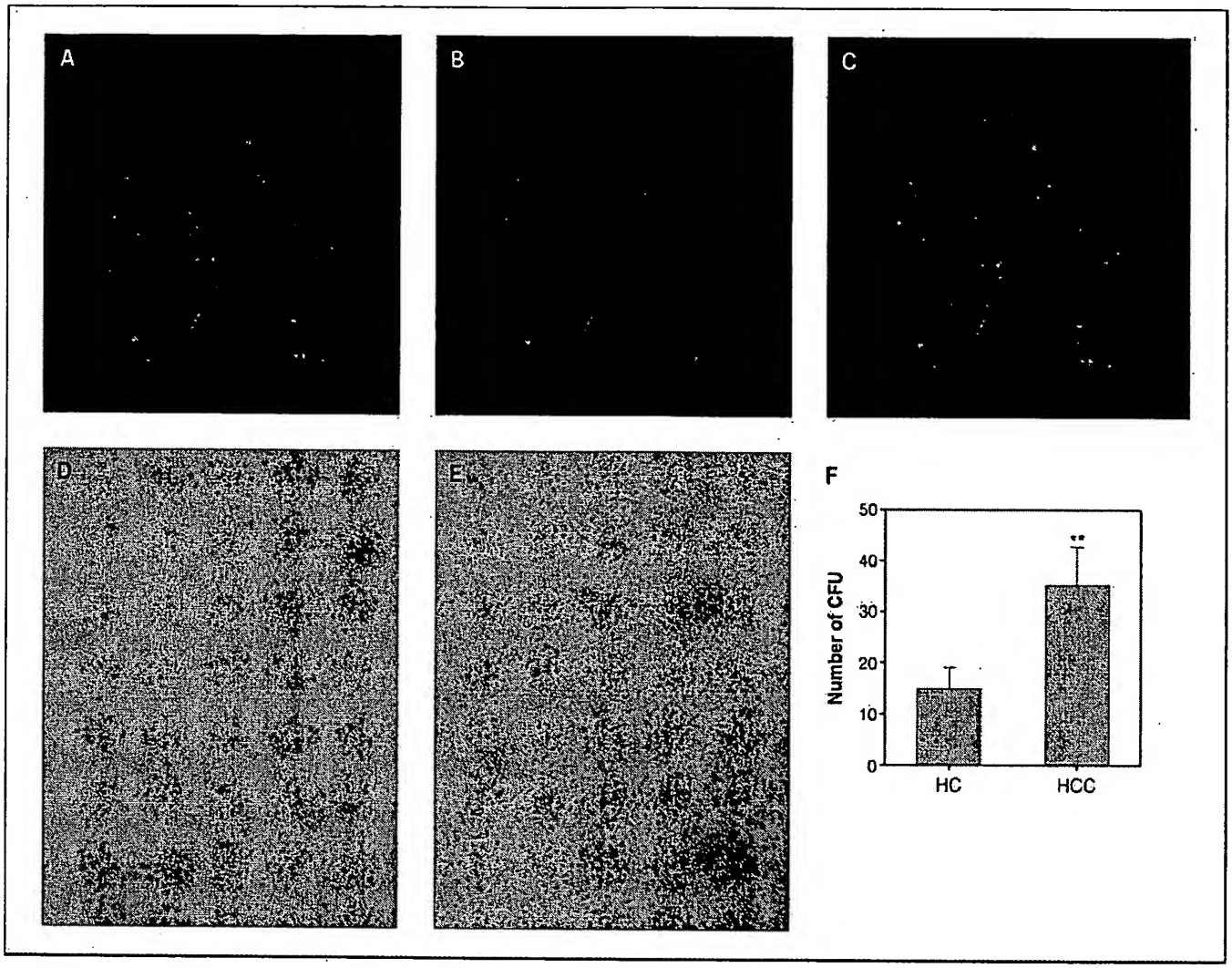

Fig. 1. Identification of EPCs *in vitro* and comparative analysis. Peripheral blood mononuclear cells were cultured and identified under fluorescent microscope on the 7th day for Ulea-1 binding (*A, green*) and 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein uptake (*B, red*). Double-positive cells, appearing in yellow in the overlay (*C*), were identified as differentiating EPCs. Representative CFUs from a HC (*D*) and a patient with HCC (*E*) were shown on the 7th day of the culture. Magnification, ×40. *F*, number of CFUs per 1 $\times 10^6$ peripheral blood mononuclear cells in HC ($n = 5$) and patients with HCC ($n = 5$). Columns, number of CFUs; bars, SD. **, $P < 0.01$.

$AC133^+$ cells that were separated from adjacent microvessels, tumor cells, and connective elements were counted as one microvessel, irrespective of the presence of a vessel lumen. The mean microvessel count of the five most vascular areas was taken as the MVD, which was expressed as the absolute number of microvessels per 0.74 mm^2 (×200 field). $PCNA^+$ cells were counted from representative areas of the sections and expressed as a percentage. The intensity of immunostaining was categorized as follows: -, negative; +, low; and ++, moderate-high.

Real-time PCR

RNA isolation and reverse transcription. Acid guanidine thiocyanate-phenol-chloroform extraction was used to isolate total RNA from liver tissues. Total RNA of peripheral blood was extracted from peripheral blood mononuclear cells by Trizol reagent (Life Technologies) according to the manufacturer's instructions. With random hexamer primers, the maximum allowed volumes of RNA samples were transcribed with ExScript RT reagent kit (TaKaRa) according to the manufacturer's protocol. RNA samples without reverse transcription were used as negative controls.

PCR. Primers and probes for human *AC133*, *CD34*, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes were designed with Primer Express 2.0 software (Applied Biosystems) and synthesized by Genecore. The basic information on primers and probes, including gene name, National Center for Biotechnology Information reference, forward primer, reverse primer, probe and its location between two exons, and product size (bp), respectively, are the following: *AC133*, NM_006017, GACCGACTGAGACCCAACATC, AGGTGCTGTTCATGTTCTCCAA, FAM-CAACAGCGATCAAGG-MGB, 7 and 8, and 103; *CD34*, NM_001025109, CCTTGCAACATCTCCCACTAAAC, TTCACTTCTCTGATGCCTGAACA, FAM-ACATCAAGGCAGAAAT-MGB, 3 and 4, and 96; and *GAPDH*, NM_002046, GGGCTGCTTTTAACTCTGGTAAAG, CCATGGGTGGAATCATATTGG, FAM-CCTCAACTACATGGTTTAC-MGB, 1 and 2, and 103. For the amplification of *AC133*, *CD34*, and *GAPDH* genes, real-time PCR was done in triplicate for each sample in a 20 μL reaction mixture, which consisted of template DNA (2 μL), primers (900 nmol/L), probe (250 nmol/L), Mg^{2+} (5 mmol/L), and Ex Taq HS (0.1 units/μL; ExScript Real-time PCR Kit, TaKaRa). PCR was done in a Stratagene Mx3005P instrument using the following thermal cycles: one cycle of 10 s at 95°C, 55 cycles of

5 s at 95°C, and 20 s at 60°C. Amplification efficiency of each individual sample was calculated by version 7.0 of LinRegPCR program (a gift from C.R. Ramakers,[3] Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands). According to the method tested by Pfaffl (32), the relative expression ratio of a targeted gene was calculated based on efficiency and the C_t compared with a reference gene (*GAPDH*).

Measurement of plasma and tumor cytosolic $VEGF_{165}$ and PDGF-BB protein concentration

The isolation of plasma and tumor cytosolic proteins was done as described by Poon et al. (15). Before surgery, peripheral venous blood samples were taken from the patients and centrifuged at 3,000 rpm for 10 min and then stored at -80°C. Protein cytosolic fractions were obtained by homogenization of tissues. Homogenates were lysed with equal volumes of radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.02% NaN_3, 1% Triton X-100, 1% SDS] with Cocktail protease inhibitor (1:200; Sigma) on ice for 30 min and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatants were assayed for cytosolic $VEGF_{165}$, PDGF-BB, and total protein concentration.

$VEGF_{165}$ and PDGF-BB in plasma and liver tissue were quantified by ELISA ($VEGF_{165}$ and PDGF-BB Immunoassay, Lifekey Corp.). Each measurement was made in duplicate, and the $VEGF_{165}$ and PDGF-BB levels were determined from a standard curve generated for each set of samples assayed. The total protein concentration in tissues was determined with the Coomassie plus protein assay reagent (Pierce Chemical Corp.). To correct for variation in total protein concentration, the relative concentrations of $VEGF_{165}$ and PDGF-BB in tissues were calculated by dividing $VEGF_{165}$ and PDGF-BB concentrations by the total protein concentration in the tissue.

Clinicopathologic database and follow-up

All clinicopathologic data were assembled prospectively in a computerized database, and all patients were followed and monitored regularly for tumor recurrence by α-fetoprotein level (monthly) and chest X-ray, together with B ultrasonic or computed tomography scan (every 3 months). The median follow-up time of all patients was 14 months (range, 3-34 months). A diagnosis of recurrence was based on typical imaging appearance in computed tomography scan and an elevated α-fetoprotein level and, if necessary, fine-needle aspiration cytology. All of the patients were followed until death or until the study closing date of October 1, 2006.

Statistical analysis

Data were expressed as mean ± SD with the range given in parentheses. Statistical comparisons were done using the *t* test, ANOVA, and linear regression when data were normally distributed. The Pearson χ^2 test was used to compare the results of two or more subgroups. All statistical procedures were done using SPSS (version 11.5; SPSS, Inc.). Values of $P < 0.05$ were considered statistically significant.

Results

Patient data. In 64 patients (53 males and 11 females; median age, 51 years) who underwent curative resection (57 cases for regular hepatectomy and 7 for orthotopic liver transplantation), the average tumor size was 6.65 ± 4.17 cm (range, 0.8-20 cm). Liver cirrhosis was detected in 60 patients; the remaining 4 patients had chronic hepatitis. The etiologies of underlying liver diseases were hepatitis B in 56 patients, hepatitis C in 1 patient, mixed viral infection in 1 patient, alcoholic cirrhosis in 4 patients, and cirrhosis of unidentified etiology in 3 patients. According to International Union Against Cancer recommendations (2002; ref. 33), 25 patients were classified as stage I, 12 patients as stage II, 26 patients as stage III, and 1 patient as stage IV. Fifty-two patients were in child's class A, 11 in class B, and 1 in class C.

Mobilized EPCs are increased in the peripheral blood of patients with HCC compared with HC. To date, no clear definition of EPCs exists and their extremely low number makes isolation difficult. Therefore, based on recent investigations (34), we determined the number of circulating EPCs by a functional assay (colony formation, 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein uptake and for Ulea-1 binding) and quantitative analysis of phenotypes (fluorescence-activated cell sorting and real-time PCR).

First, circulating EPCs were evaluated in the peripheral blood of patients with HCC and HC by colony-forming assay. Representative CFUs were observed and counted on the 7th

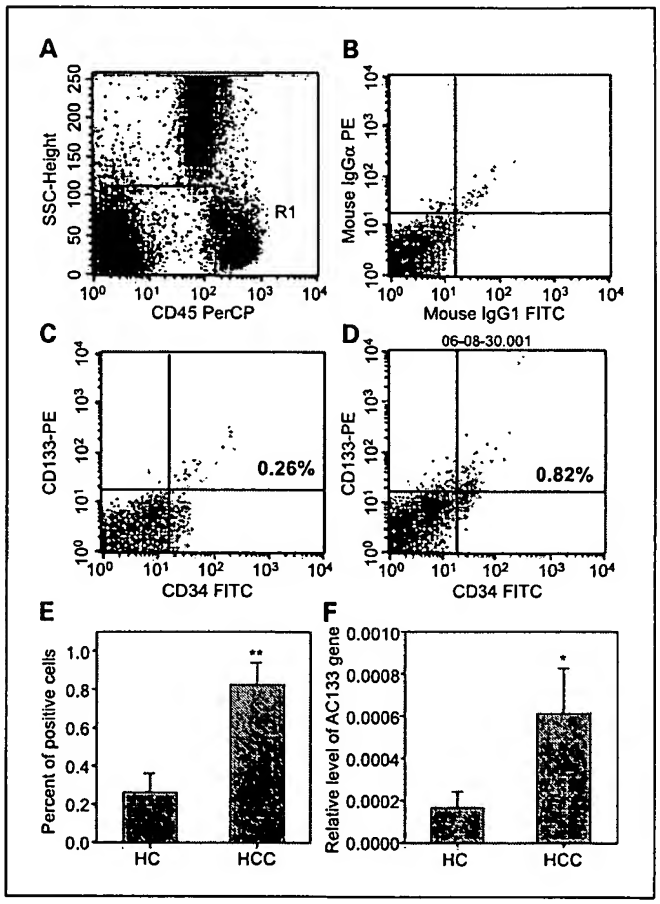

Fig. 2. Frequency of $AC133^+$ and $CD34^+$ cells in patients with HCC compared with HC. *A*, forward and side scatter evaluation with lack of the hematopoietic marker CD45 was used to generate the analysis gate. *B*, negative controls stained with phycoerythrin- or FITC-labeled IgG isotype controls. The percentage of the population expressing the indicated double antigens ($AC133^+$ and $CD34^+$) from HC (*C*) and patients wit HCC (*D*) was shown in each quadrant. *E*, the percentage of $CD34^+$ and $AC133^+$ cells in HC ($n = 6$) and patients with HCC ($n = 11$). *F*, the relative level of *AC133* gene in peripheral blood of HC ($n = 5$) and patients with HCC ($n = 12$). Columns, percentage of positive cells (*E*) and relative level of *AC133* gene (*F*); bars, SD. *, $P < 0.05$; **, $P < 0.01$.

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day. First, EPCs were identified as adherent cells that were positive for both 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein uptake and Ulea-1 binding as determined by fluorescent microscopy (Fig. 1A-C). Representative EPC colonies were characterized by a central cluster of round cells surrounded by radiating, thin, flat, elongated cells (Fig. 1D, from a HC; Fig. 1E, from a patient with HCC). Relative to HC (15.00 ± 4.06, $n = 5$), mean CFU scores from HCC patients (35.20 ± 7.56, $n = 5$) were higher on the 7th day ($P = 0.001$; Fig. 1F).

Moreover, the mean percentage of $CD34^+$ and $AC133^+$ cells in the peripheral blood of patients with HCC (0.82% ± 0.12, $n = 11$) was elevated relative to HC (0.26% ± 0.10, $n = 6$; $P = 0.002$; Fig. 2A-E). In addition, the relative level of *AC133* gene expression in the peripheral blood of patients with HCC (0.00061 ± 0.00021, $n = 12$) was higher than in HC (0.00017 ± 0.00008, $n = 5$; $P = 0.039$; Fig. 2F). In conclusion, there are more mobilized EPCs in the peripheral blood of patients with HCC than in HC.

$AC133^+$ and $CD34^+$ cells incorporate into vessels in tumor tissue. In frozen sections from 10 different tumor specimens, cells expressing AC133 and CD34 antigens were simultaneously identified in endothelium by direct immunofluorescence staining using anti-AC133 and CD34 antibodies. In tumor tissue, all of the $AC133^+$ cells were positive for the CD34 antigen in portal veins and microvessels. The double-positive cells were incorporated into vessel walls of different sizes, such as portal veins (Fig. 3A and B) and microvessels (Fig. 3B and C) in tumors. Additionally, a projection made from segmented confocal image data indicated that $AC133^+$ and $CD34^+$ cells incorporated into portal veins and microvessels in tumor tissue (Fig. 3D-I).

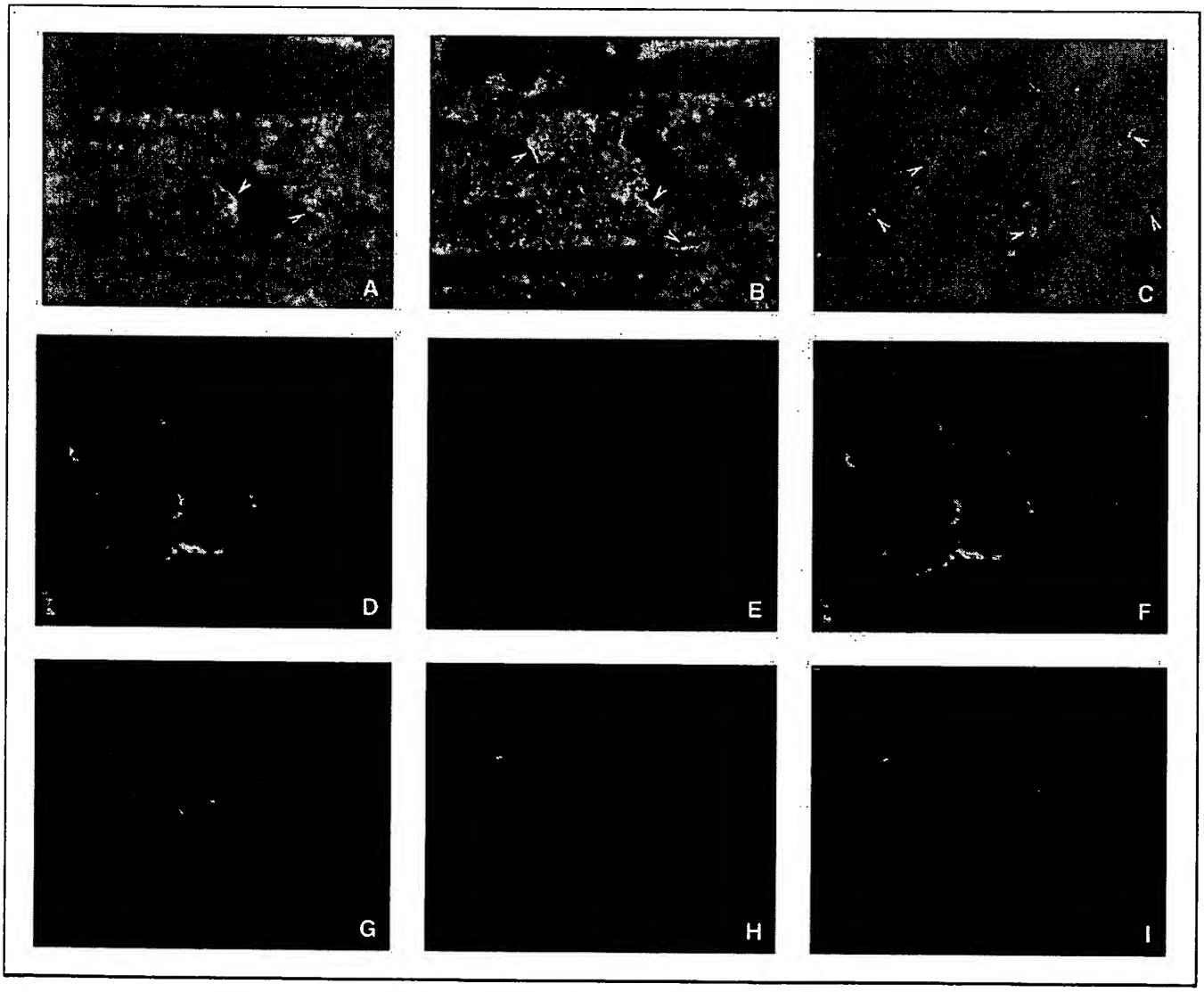

Fig. 3. Location of $AC133^+$ and $CD34^+$ cells in liver cancer. Frozen sections of tumor specimens labeled with FITC-conjugated anti-CD34 (*green*) and phycoerythrin-conjugated anti-AC133 (*red*). *A* to *C*, representative merged images showed that all of $AC133^+$ cells are localized $CD34^+$ endothelium, such as portal veins (*A* and *B*) and microvessels (*B* and *C*) in tumor tissue. Arrows, double-positive cells (*yellow*). Two confocal Z series were shown (*D-F*, portal vein; *G-I*, microvessels). *D* and *G*, FITC-labeled CD34 binding (*green*) specifically to the tumor vasculature; *E* and *H*, phycoerythrin-AC133 antibody binding (*red*) within those same cells; *F* and *I*, merged image of the two previous images, showing colocalization of CD34 and AC133 (*yellow*) to the vessels. Magnification, ×200.

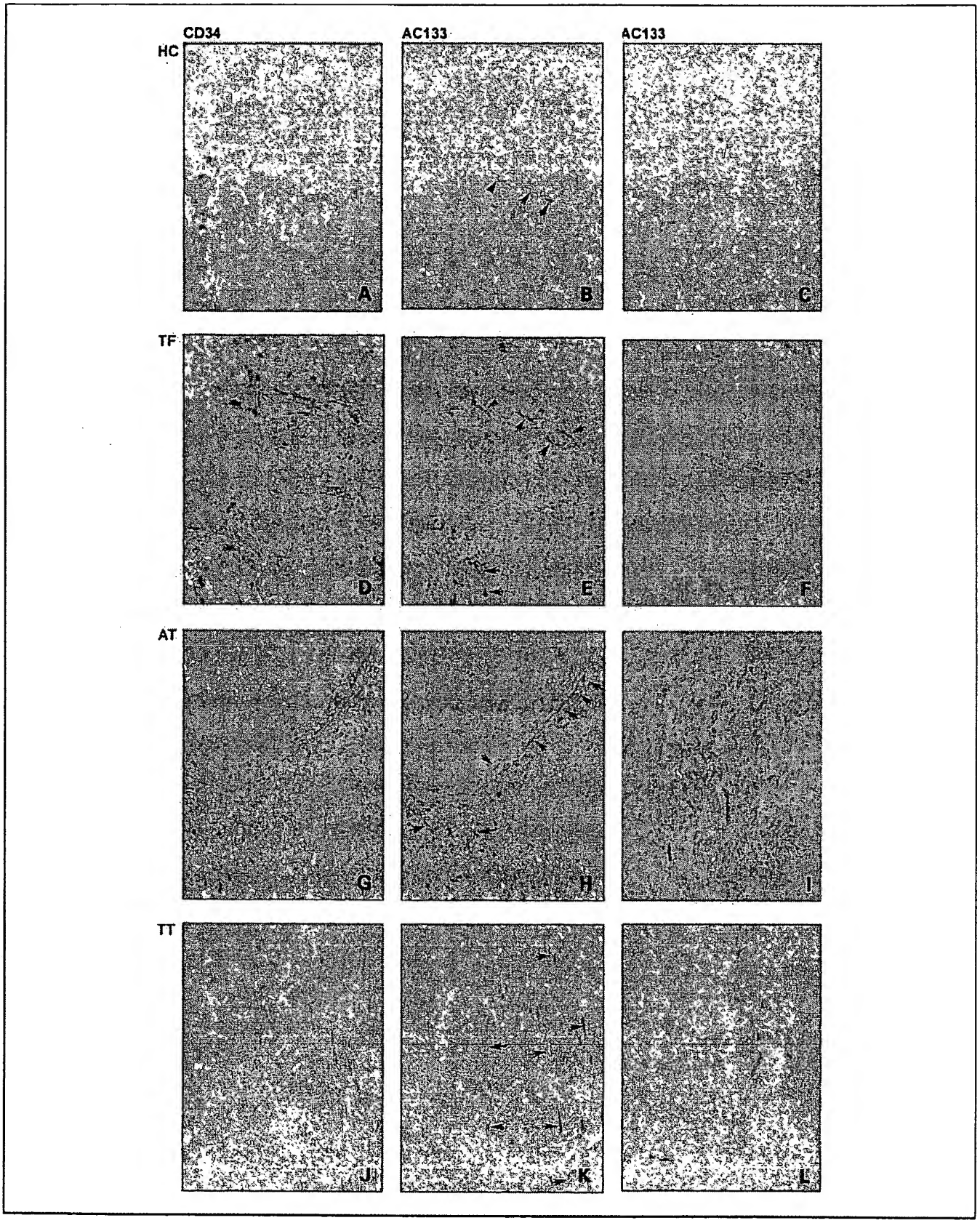

Fig. 4. Distribution of AC133 and CD34 antigen in HC, TT, AT, and TF. Representative immunostaining images were presented for CD34 (*A, D, G,* and *J*) and AC133 (*B, E, H,* and *K*) in two consecutive 4-μm sections of HC (*A* and *B*), TF (*D* and *E*), AT (*G* and *H*), and TT (*J* and *H*), respectively. Arrows, double-positive cells. $AC133^+$ cells incorporating into microvessels in HC, TF, AT, and TT were presented, respectively (*C, F, I,* and *L*). Magnification, ×200.

EPCs in patients with HCC were recruited and incorporated into the microvessels in TF, AT, and TT and were especially abundant in AT. In samples from HC, consecutive sections stained for the AC133 and CD34 antigens showed that the $AC133^+$ and $CD34^+$ cells were incorporated mostly into the vessels of portal areas (Fig. 4A-C). $AC133^+$ and $CD34^+$ cells were incorporated into vessels of different sizes, most frequently in microvessels in samples of cirrhotic and tumor tissue in TF (Fig. 4D-E), AT (Fig. 4G-H), and TT (Fig. 4J-K). Highlighted microvessels ($AC133^+$) showed two patterns of expression in TF, AT, and TT sections. The first showed branching; in the second, the microvessels were small without apparent lumina (endothelial sprouts; Fig. 4F, I, and L). Of note, there were some $AC133^+$ cells in stroma septa (Fig. 4H). Compared with AC133-MVD in normal liver samples (9.00 ± 0.54, n = 4), increased AC133-MVD (microvessels/0.74 mm^2) was found in 18 patients with HCC within TT (53.56 ± 10.56), AT (84.76 ± 11.32), and TF (48.33 ± 7.79) samples. As determined with a paired t test (n = 18), AC133-MVD in AT samples was the highest among TF, AT, and TT samples, whereas CD34-MVD (microvessels/0.74 mm^2) in TT samples was the highest among TF (86.51 ± 25.10), AT (117.32 ± 37.81), and TT (323.29 ± 101.14) samples (Fig. 5A and B).

Compared with amounts in HC (n = 8), the relative levels of *AC133* and *CD34* gene expression were elevated in TF, AT, and TT samples (n = 64; Fig. 5C and D). As determined with a paired t test, the relative level of *AC133* gene expression in AT samples was the highest (Fig. 5C), whereas the relative *CD34* gene expression in TT samples was the highest among TF, AT, and TT samples (Fig. 5D).

As determined by protein and gene expression levels, $AC133^+$ EPCs in patients with HCC were recruited and incorporated into the microvessels in TF, AT, and TT and were especially abundant in AT.

$VEGF_{165}$ and PDGF-BB in plasma and liver of HCC for mobilization and recruitment of EPCs. The median level of preoperative plasma $VEGF_{165}$ (223.80 ± 32.19 pg/mL) and PDGF-BB (605.25 ± 128.38 pg/mL) in patients with HCC (n = 20) was higher than that of plasma $VEGF_{165}$ (24.79 ± 4.88 pg/mL; P = 0.003) and PDGF-BB (37.13 ± 10.46 pg/mL; P = 0.000) in HC (n = 15; Fig. 6A). In the peripheral blood of patients with HCC, the relative level of *AC133* gene expression correlated with plasma VEGF levels (r = 0.629; P = 0.009) and PDGF-BB (r = 0.618; P = 0.024; Fig. 6B). Thus, $VEGF_{165}$ and PDGF-BB in plasma may be key factors for the mobilization of bone marrow-EPCs into peripheral blood. In tissues, the relative concentration of $VEGF_{165}$ (P = 0.017) and PDGF-BB (P = 0.001) in HC (n = 4) was the lowest of the four groups. The relative concentration of $VEGF_{165}$ was lower in TT than in TF (P = 0.025) and AT (P = 0.024), whereas the relative concentration of PDGF-BB was higher in TT than that in TF (P = 0.004) and AT (n = 14; P = 0.006; Fig. 6C). Furthermore, there was no correlation between the relative concentration of the two factors ($VEGF_{165}$ and PDGF-BB) and the relative levels of *AC133* gene expression and MVD in the respective groups. Nevertheless, recruitment and homing of EPCs in the liver with HCC may also be affected by other proangiogenic factors besides $VEGF_{165}$ and PDGF-BB.

Relationship between the level of AC133 gene expression in tissue and clinical variables. Within the analyzed clinical variables (gender, age, total bilirubin, and glucose), the relative level of *AC133* gene expression was distributed equally in TF,

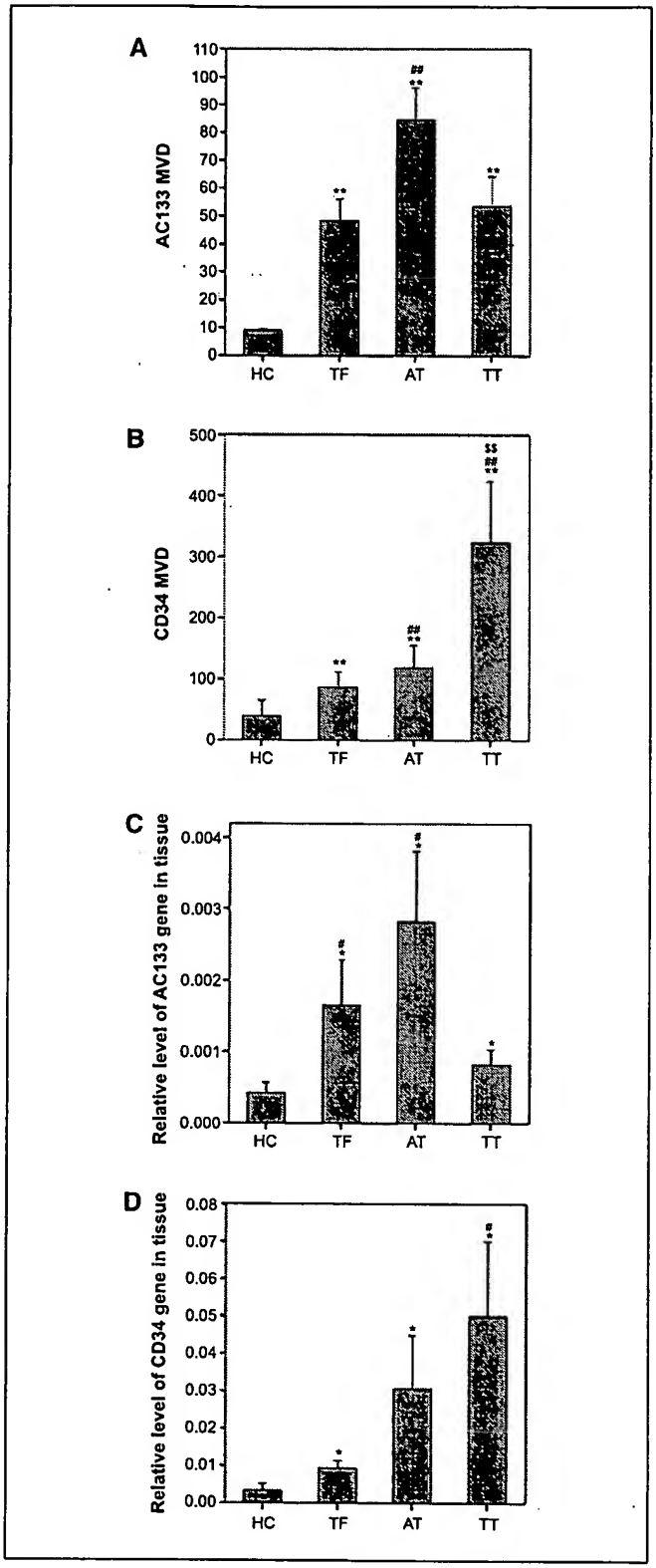

Fig. 5. Frequency of AC133 and CD34 antigens and genes in HC, TT, AT, and TF. *A*, AC133-MVD in HC (n = 4), TF, AT, and TT (n = 18) were compared. **, P < 0.01 versus HC; ##, P < 0.01 versus TF and TT. *B*, CD34-MVD in HC (n = 4), TF, AT, and TT (n = 18) were compared. **, P < 0.01 versus HC; ##, P < 0.01 versus TF; $$, P < 0.01 versus AT. *C*, relative level of *AC133* gene in HC (n = 8), TF, AT, and TT (n = 64). *, P < 0.05 versus HC; #, P < 0.05 versus TT. *D*, relative level of *CD34* gene in HC (n = 8), TF, AT, and TT (n = 64). *, P < 0.05 versus HC; #, P < 0.05 versus TF. Columns, mean; bars, SD.

AT, and TT samples. Clinicopathologic variables that correlated with the relative level of *AC133* gene expression in AT samples included platelets (≤150 × 10^9/L or >150 × 10^9/L), hepatitis B virus status, the absence of tumor capsule, venous invasion, positive frequency of PCNA (1 and 2), and early recurrence time (≤6 or >6 months). Furthermore, the relative level of *AC133* gene expression in TF sample correlated only with platelets and hepatic vein invasion, whereas the relative level of *AC133* gene expression in TT sample correlated with capsule invasion. Therefore, the level of *AC133* gene expression in AT could be used as a biomarker for predicting the progression of HCC (Table 1).

Discussion

The previous reports have indicated that EPCs can be identified by simultaneous expression of the cell surface markers CD34, AC133, and kinase insert domain-containing receptor (4, 5). In this investigation, $AC133^+$ and $CD34^+$ cells were regarded as EPCs. This is supported by the following: (*a*) AC133, the surface marker of progenitor cells, is used to evaluate the level of EPCs in circulation or in tumors such as non-small cell lung cancer (9), HCC (22), breast cancer (35), and multiple myeloma (36). (*b*) CD34-MVD has been recognized as a biomarker for tumor angiogenesis, especially in HCC. (*c*) Kinase insert domain-containing receptor is expressed not only in hepatocytes and liver cancer cells but also in endothelial cells and progenitor cells in HCC with cirrhosis present (37). In addition, kinase insert domain-containing receptor expression in liver with HCC (TT, AT, and TF) was diffuse and similar in levels.[4] A possible explanation for this observation might be that kinase insert domain-containing receptor staining is not specific to endothelial cells. (*d*) It is conceivable that $AC133^+$ cells are cancer stem cells in HCC (38), prostate cancer (39), and colon cancer (40) because they possess a marked capacity for proliferation, self-renewal, and differentiation, although they represent only a small population (~0.1-2.5%) of the cancer cells (39, 40). However, there have not been any reports that $AC133^+$ and $CD34^+$ cells are cancer stem cells. In fact, in the present investigation, $AC133^+$ and $CD34^+$ cells, which were present in the vessel walls, were classified as endothelial-like cells, not as cancer cells, by our senior pathologist following immunohistochemistry and immunofluorescent staining. Further investigation showed that $AC133^+$ cells were negative for hepatocyte paraffin-1 antigen, which is the specific marker of hepatocytes or cancer cells in HCC.[5]

Thus far, the clinical significance of circulating EPCs has been considered for non-small cell lung cancer (9), HCC (22), and breast cancer (35). Especially in breast cancer, a surrogate biomarker approach involving measurement of circulating EPCs has been used to determine the optimal dose of antiangiogenic drugs (35). In the current investigation, the relative levels of *AC133* gene and antigen expression were higher in the peripheral blood of patients with HCC compared with HC, which positively correlated with plasma $VEGF_{165}$, PDGF-BB, and some clinicopathologic variables.[5] In Poon et al. study, CFU scores, which correlated with the levels of VEGF and interleukin-8,

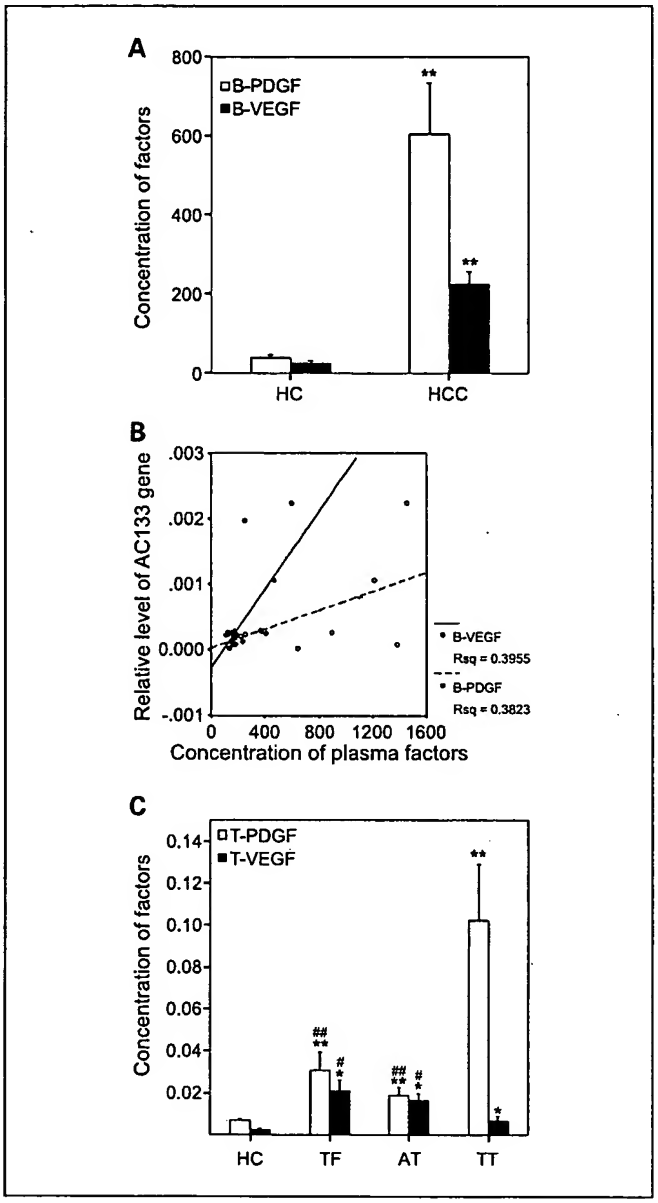

Fig. 6. Concentration of $VEGF_{165}$ and PDGF-BB in plasma and tissue. *A*, plasma $VEGF_{165}$ and PDGF-BB level in the HC (n = 15) and patients with HCC (n = 20). **, $P < 0.01$. *B*, correlation of the relative level of *AC133* gene with plasma $VEGF_{165}$ and PDGF-BB level in peripheral blood of patients with HCC (n = 12). *C*, the relative concentration of $VEGF_{165}$ and PDGF-BB in HC (n = 4), TF, AT, and TT (n = 14). *, $P < 0.05$; **, $P < 0.01$ versus HC; #, $P < 0.05$; ##, $P < 0.01$ versus TT. Columns and points, mean; bars, SD.

were elevated in patients with unresectable HCC compared with patients with early resectable HCC or liver cirrhosis and HC (22). Thus, EPCs were mobilized from bone marrow in HCC patients. The surface marker AC133 and CFU scores may be valuable biomarkers to predict progression of HCC.

It is generally accepted that vascularization of tumors arises exclusively from endothelial sprouting. Arbab et al. (6) and Shirakawa et al. (7) used mouse tumor models to show that bone marrow-derived EPCs are also involved in tumor vasculogenesis, especially in the surrounding of the tumor. Until recently, there have been only two clinical reports that have evaluated the participation of EPCs in the progression of solid

[4] Unpublished data.

[5] Data unpublished due to limitations in manuscript length.

tumors (8, 9). In the current investigation, EPCs were incorporated into vessel walls of different sizes, mostly in the microvessels in cirrhotic and tumor tissues of patients with HCC, although only a few EPCs were found in the portal area vessels in normal liver samples. Proangiogenic factors, such as VEGF (41) and PDGF (42), which have the strong expression in HCC and cirrhotic tissue samples, may regulate not only endothelial cell proliferation but also promote EPC mobilization and homing into HCC and cirrhotic tissue. Furthermore, tumor stroma provides pathways for neovessels and serves as a reservoir for growth factors and other macromolecules (43). Our results agree with this concept. We found that encapsulated HCCs have more EPCs in AT and TT than nonencapsulated ones. In these tumors with capsule invasion, more EPCs were present in tumor tissue. Of note, a higher level of $VEGF_{165}$ mRNA in nonmalignant liver tissue correlated significantly with a higher risk of HCC recurrence and recurrence-related mortality, vascular permeation, daughter nodules, cellular dedifferentiation, and absent or incomplete capsule (44). In present investigation, the relative level of *AC133* gene expression in AT also correlated with clinicopathologic variables (platelets, HBsAg status, the absence of tumor capsule, venous invasion, positive frequency of PCNA, and early recurrence time). Therefore, mobilized EPCs participate in tumor vasculogenesis of HCC. *AC133* gene or antigen in AT may be used as an angiogenic biomarker predicting the progression of HCC.

Thus far, there are no published investigations of the enrichment of EPCs in nonmalignant liver tissue, especially in tissue adjacent to tumors. The basis for the presence of more EPCs in microvessels in tissue adjacent to tumors is not known. The recruitment and homing of EPCs are affected by hypoxia, angiogenic factors, and adherent molecules (45). In this regard, the particular pathology in HCC with liver cirrhosis may be important.

HCC is a cancer associated in most cases with chronic liver disease, such as chronic viral hepatitis and cirrhosis, especially in Southeast Asia. The nonmalignant liver itself has a precancerous change with angiogenesis. During liver cirrhosis, fibrogenesis induces intrahepatic shunts and a barrier between the sinusoids and the hepatocytes (46). In addition, hepatitis B

Table 1. The relationship between the relative level of *AC133* gene in TF, AT, and TT and clinical variables

Clinical variables	Class	No.	*AC133* in TF		*AC133* in AT		*AC133* in TT	
			Mean	*P*	Mean	*P*	Mean	*P*
Platelets (10^9/L)	<150	41	0.00139	0.002*	0.00163	0.003*	0.00072	0.385
	≥150	23	0.00529		0.00504		0.00010	
HBV infection	No	8	0.00362	0.328	0.00746	0.001*	0.00041	0.234
	Yes	56	0.00267		0.00207		0.00088	
AFP	<363	30	0.00239	0.370	0.00331	0.533	0.00066	0.271
	≥363	34	0.00314		0.00235		0.00096	
Cirrhosis	No	4	0.00106	0.528	0.00004	0.388	0.00142	0.084
	Yes	60	0.00291		0.00295		0.00078	
TNM classification	I-II	37	0.00279	0.344	0.00357	0.827	0.00093	0.840
	III-IV	27	0.00400		0.00392		0.00101	
Tumor size (cm)	<2	7	0.00106	0.798	0.00106	0.738	0.00020	0.450
	≥2, <5	17	0.00276		0.00256		0.00114	
	≥5	40	0.00311		0.00333		0.00079	
Lesion	Solitary	40	0.00219	0.292	0.00325	0.184	0.00084	0.492
	Multiple	24	0.00379		0.00181		0.00079	
Node metastasis	No	55	0.00197	0.418	0.00289	0.570	0.00069	0.226
	Yes	9	0.00130		0.00180		0.00061	
Necrosis in mass	No	35	0.00330	0.157	0.00301	0.260	0.00074	0.736
	Yes	29	0.00218		0.00255		0.00091	
Portal vein invasion	No	40	0.00253	0.841	0.00106	0.003*	0.00097	0.071
	Yes	24	0.00322		0.00576		0.00056	
Hepatic vein invasion	No	42	0.00130	0.002*	0.00143	0.000*	0.00065	0.082
	Yes	22	0.00563		0.00602		0.00113	
Hepatic capsule invasion	No	29	0.00150	0.064	0.00162	0.061	0.00073	0.679
	Yes	35	0.00386		0.00396		0.00089	
Capsule invasion	No	43	0.00200	0.142	0.00251	0.588	0.00061	0.024†
	Yes	21	0.00458		0.00363		0.00131	
Tumor capsule	No	21	0.00117	0.096	0.00082	0.022†	0.00070	0.460
	Yes	43	0.00366		0.00429		0.00094	
Differentiation	Low	13	0.00125	0.166	0.00092	0.221	0.00064	0.494
	Mid-high	51	0.00330		0.00272		0.00090	
PCNA (intensity)	1	23	0.00401	0.077	0.00071	0.035†	0.00079	0.733
	2	41	0.00211		0.00383		0.00083	
Recurrence time (mos)	<6	18	0.00311	0.921	0.00615	0.000*	0.00090	0.059
	≥6	46	0.00267		0.00137		0.00061	

Abbreviations: HBV, hepatitis B virus; AFP, α-fetoprotein; TNM, tumor-node-metastasis.
*$P < 0.01$.
†$P < 0.05$.

virus X protein increases the transcriptional activity and protein level of hypoxia-inducible factor-1α, thereby promoting angiogenesis during hepatocarcinogenesis (47). Regeneration in the cirrhotic liver would pose a potential of malignant degeneration and correlated with serum VEGF level (48). Moreover, VEGF expression is also modulated by inflammatory cytokines released from infiltrating inflammatory cells in surrounding cirrhotic liver tissues (25). It is developing into an angiogenetic environment that may secrete higher proangiogenic factors. More and more investigations reported proangiogenic factors, such as VEGF (25), hepatic growth factor (26), and inducible nitric oxide (49), have higher expression in the surrounding liver than in tumors, which were consistent with our results. In addition, our further investigations have indicated that the expression of CD105, hypoxia-inducible factor-1α, and vascular cell adhesion molecule-1 are elevated in AT compared with TF and TT (parts of data to be published in another article about the distribution of CD105 in HCC). So it is concluded that the recruitment and homing of EPCs into AT may be affected by hypoxia-inducible factor-1α, proangiogenic factors, and cell-matrix adhesion molecules resulting from both liver cirrhosis and HCC. The exact mechanism on recruitment and homing of EPCs into liver cirrhosis and cancer is worthy of further investigation.

In summary, our data indicate that (*a*) EPCs were mobilized into the peripheral blood of patients with HCC, and this mobilization correlated with plasma $VEGF_{165}$ and PDGF-BB; (*b*) EPCs were incorporated into vessel walls of different sizes and were found primarily in the microvessels in cirrhotic and malignant liver specimens; (*c*) the relative level of *AC133* gene expression in AT correlated with clinicopathologic variables, such as platelets ($\leq 150 \times 10^9$/L or $>150 \times 10^9$/L), hepatitis B virus status, the absence of a tumor capsule, hepatic or portal vein invasion, positive frequency of PCNA (1 and 2), and early recurrence time (≤6 or >6 months). These findings suggest that mobilized EPCs participate in the vasculogenesis of HCC and may serve as biomarkers for predicting the progression of HCC. As EPCs are endowed with the capacity to home the tumor vasculature, they might be used to deliver drugs. In addition, the recruitment and distribution of EPCs in HCC was different from other cancers. Therefore, the identification of chemokines/cytokines and tissue-specific extracellular matrix components that are involved in the recruitment of EPCs in HCC might provide new targets for the treatment of HCC.

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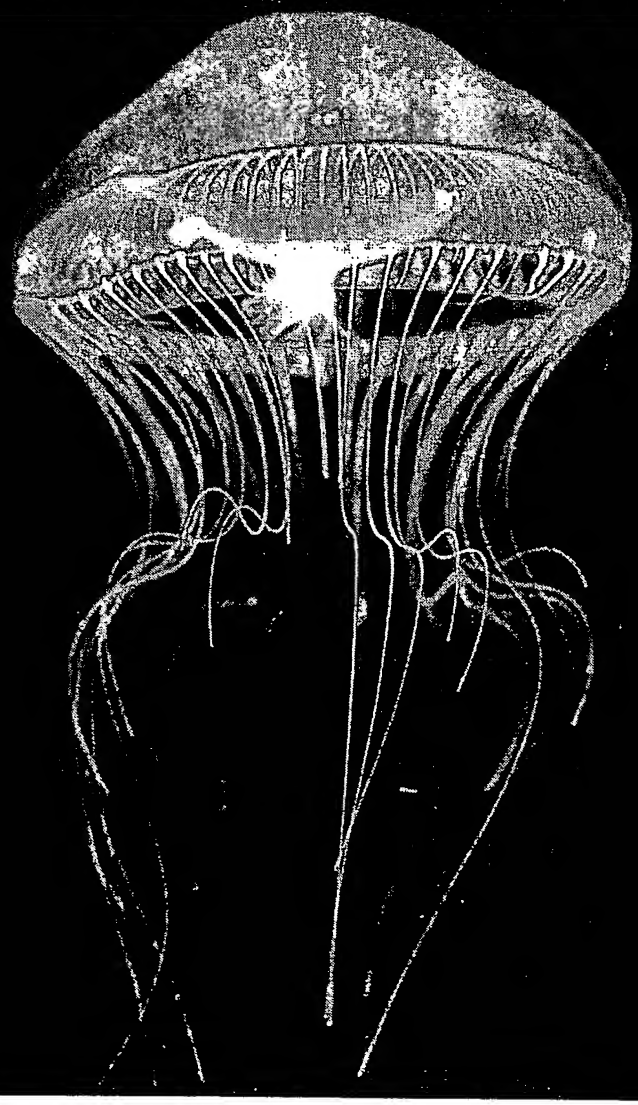

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A TYPICAL MAMMALIAN CELL CONTAINS ~10^{-5} µg OF RNA, 80–85% of which is ribosomal RNA (chiefly the 28S, 18S, 5.8S, and 5S species). Most of the remaining 15–20% consists of a variety of low-molecular-weight species (e.g., transfer RNAs and small nuclear RNAs). These abundant RNAs are of defined size and sequence and can be isolated in virtually pure form by gel electrophoresis, density gradient centrifugation, anion-exchange chromatography, or high-performance liquid chromatography (HPLC). By contrast, messenger RNA, which makes up between 1% and 5% of the total cellular RNA, is heterogeneous in both size — from a few hundred bases to many kilobases in length — and sequence. However, most eukaryotic mRNAs carry at their 3′ termini a tract of polyadenylic acid residues that is generally long enough to allow mRNAs to be purified by affinity chromatography on oligo(dT)-cellulose. The resulting heterogeneous population of molecules collectively encodes virtually all of the polypeptides synthesized by the cell.

Because ribose residues carry hydroxyl groups in both the 2′ and 3′ positions, RNA is chemically much more reactive than DNA and is easy prey to cleavage by contaminating RNases — enzymes with various specificities that share the property of hydrolyzing diester bonds linking phosphate and ribose residues. Because RNases are released from cells upon lysis and are present on the skin, constant vigilance is required to prevent contamination of glassware and bench tops and the generation of RNase in aerosols. The problem is compounded since there is no simple method to inactivate RNases. Because of the presence of intrachain disulfide bonds, many RNases are resistant to prolonged boiling and mild denaturants and are able to refold quickly when denatured. Unlike many DNases, RNases do not require divalent cations for activity and thus cannot be easily inactivated by the inclusion of ethylenediaminetetraacetic acid (EDTA) or other metal ion chelators in buffer solutions. The best way to prevent problems with RNase is to avoid contamination in the first place (please see the information panels on HOW TO WIN THE BATTLE WITH RNASE, INHIBITORS OF RNASES, and DIETHYLPYROCARBONATE at the end of this chapter).

This chapter is divided into two parts (please see Figure 7-1). The first series of protocols (Protocols 1 through 6) is devoted to the isolation and purification of total RNA and, subsequently, of poly(A)$^+$ RNA.

The second series of protocols (Protocols 7 through 12) deals with various approaches for the analysis of purified RNA, in particular for assessing gene expression and/or gene structure. Hybridization by northern transfer (Protocols 7 and 8) or by dot/slot blotting (Protocol 9) may be used to determine the size and abundance of a particular species of RNA. Details of the fine structure of a particular transcript may be assessed by S1 mapping or ribonuclease protection (Protocols 10 and 11). The use of either of these techniques allows the detection of the 5′ and 3′ ends of a particular mRNA, as well as the splice junctions, precursors, and processing intermediates of mRNA. Primer extension (Protocol 12) provides a measure of the amount of a particular mRNA species and allows an exact determination of the 5′ end of the mRNA.

Work is of two kinds: first, altering the position of matter at or near the earth's surface relatively to other such matter; second, telling other people to do so. The first is unpleasant and ill paid; the second is pleasant and highly paid.

Bertrand Russell

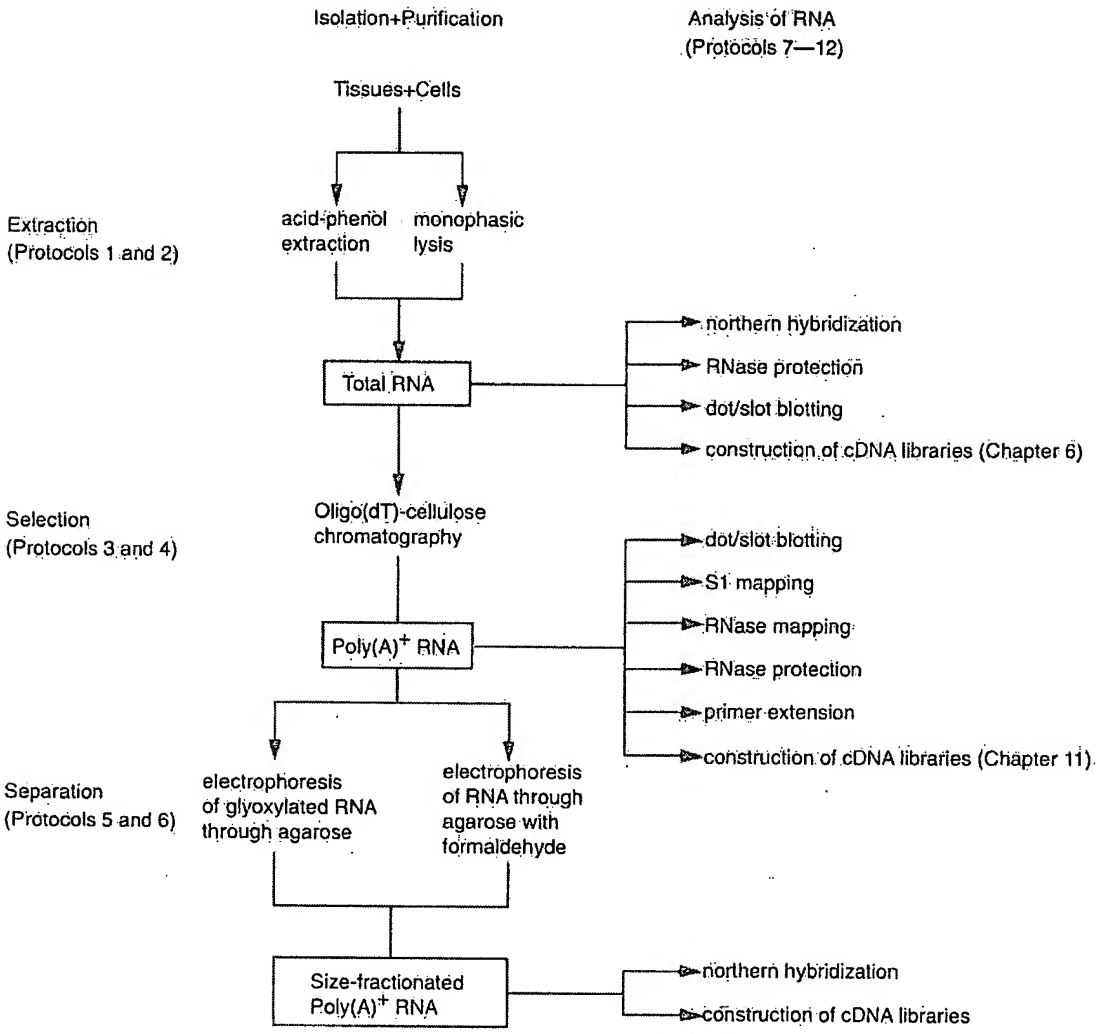

FIGURE 7-1 Flowchart of Methods

Protocol 3

Selection of Poly(A)$^+$ RNA by Oligo(dT)-Cellulose Chromatography

BY CONTRAST TO rRNA, 5S RNA, 5.8S RNA, AND tRNA, most eukaryotic mRNAs carry tracts of poly(A) at their 3′ termini. mRNAs can therefore be separated from the bulk of cellular RNA by affinity chromatography on oligo(dT)-cellulose (Edmonds et al. 1971; Aviv and Leder 1972). The method takes advantage of the ability of poly(A) tails on the mRNAs to form stable RNA-DNA hybrids with short chains of oligo(dT) (generally 18–30 nucleotides in length) linked to a supporting cellulose matrix (please see the panel on OLIGO(dT) CELLULOSE on the following page). Because only a few dT-A base pairs are formed, high salt must be added to the initial chromatography buffer to stabilize the nucleic acid duplexes. After nonpolyadenylated RNAs have been washed from the matrix, a low-salt buffer is used to destabilize the double-stranded structures and to elute the poly(A)$^+$ RNAs from the resin.

Poly(A)$^+$ RNA can be selected by chromatography on oligo(dT) columns (this protocol) or by batch elution (Protocol 4). Column chromatography is the preferred method for purification of large quantities (>25 µg) of nonradioactive poly(A)$^+$ RNA extracted from mammalian cells. For simultaneous processing of many samples of mammalian RNA, whether radioactive or not, batch elution is the better choice because fewer fractions are collected, which speeds up the process; and because a finer grade of oligo(dT)-cellulose (type III) can be used, which increases the efficiency of binding and elution of RNA. In general, between 1% and 10% of the total RNA applied to an oligo(dT) column is recovered as poly(A)$^+$ RNA. However, it is very difficult to remove all of the nonpolyadenylated RNA species completely, even after five to six cycles of affinity chromatography.

Oligo(dT)-cellulose chromatography represents an essential step when preparing mRNA to be used as a template for construction of cDNA libraries. In addition, poly(A)$^+$ RNA usually yields better results than total RNA when analyzed by blot hybridization, PCR, or nuclease S1 and RNase protection assays. This improvement is attributable to the 10–30-fold purification of mRNA obtained by chromatography on oligo(dT)-cellulose.